A Dissertation
for the Degree of Master of Science

Validation of particulate TLR agonists as vaccine adjuvants for immunization efficiency of M5BT, multi-epitope subunit vaccine against FMD

구제역 바이러스 방어를 위한 멀티-에피톱 아단위 백신 M5BT의 면역 증강제로서 미립자화 TLR 아고니스트의 효과 검증

February, 2017

By
Eojin Jung

Department of Agricultural Biotechnology
Graduate School
Seoul National University

Summary

Multi-epitope vaccine is one of the strategies to cope with Foot-and-mouth disease which has significant global impact. However, low immunogenicity derived from its protein structure requires aids of adjuvant. CpG ODN and poly I:C are agonists recognized by certain toll-like receptor expressed on immune cells and thereby, modulate innate and adaptive immunity.

In this study, first, modified medium was suggested for vaccine production. It was confirmed *E.coli* cultured in modified terrific broth-2 (T2B) showed improved cell and protein yield.

Next, the synergistic adjuvant effect of CpG ODN and poly I:C was evaluated by cell viability, cytokine release and antigen-specific T and B cell immune response through particle formation between vaccine and adjuvant via ionic interaction. Consequently, the adjuvanticity of two TLR agonists had positive effects on cell proliferation, pro-and anti-inflammatory cytokine increase and elicited desirable T and B cell immunity.

Therefore, this study was suggested as advanced subunit vaccine strategy combined with efficient culture system and adjuvant partner against FMD infection.

Keywords : Foot-and-mouth disease. Adjuvant. Multi-epitope vaccine. TLR agonist, CpG ODN, poly I:C, synergistic effect

Student number : 2015-21794

Contents

List of Tables and Figures

<Table>

<Figure>

List of Abbreviations

αMEM: alpha minimum essential medium including ribonucleosides, deoxyribionucleosides and 2 mM L-glutamine

ACK: Ammonium chloride-potassium

BSA: Bovine serum albumin

CFA: Complete Freund's adjuvant

CpG ODNs: CpG oligodeoxynucleotides

DEPC: Diethyl pyrocarbonate

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

FBS: Fetal bovine serum

FE-SEM: Field-emission scanning electron microscope

HRP: Horseradish peroxidase

IFA: Incomplete Freund's adjuvant

IPTG: Isopropyl β-D-1-thiogalactopyranoside

IRF5: Interferon regulatory factor 5

LB: Lysogeny broth

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

$O.D_{600}$: optical density at a wavelength of 600 nm

PBS: Phosphate-buffered saline

PTA: Sodium (K) phosphotungstate

qRT-PCR: Quantitative real-time polymerase chain reaction

rmGM-CSF: Recombinant mouse granulocyte-macrophage colony stimulating factor

RPMI 1640: Roswell Park Memorial Institute 1640

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

T1B: Modified terrific broth - 1

T2B: Modified terrific broth - 2

TB: Terrific broth

TEM: Transmission electron microscopy

TRizol: Guanidinium-thiocyanate-phenol-chloroform

I. Introduction

In recent years, the economic losses because of worldwide Foot-and-mouth (FMD) outbreaks hampered the development of livestock industry. Despite the increased requirement for vaccine against FMD virus, wide geographical serotype distribution, broad antigenic diversity and rapid transmission made hard to develop efficient vaccine.

Thus, subunit vaccine strategy using multiple epitopes recognized by T or B cells have been tried to overcome disadvantages of conventional vaccine and construct protective immunological memory in host immune system. Basically, subunit vaccine have no-risk for infection and can be easily produced in host *E.coli*. In particular, numerous studies have indicated multi-epitope vaccine using G-H loop sequences in VP1 structural protein provoked protective immunity against FMD.

To improve vaccine efficacy, approaches to utilize adjuvant system with protein-based vaccine have been shown in recent days. Basically adjuvant gives vaccine benefits by reducing antigen dose and number of vaccination, improving functional antibody titers and robust and long-lasting cell-mediated immune response.

Especially, toll-like receptors (TLRs) are regarded as pivotal immune-modulator as it is involved in non-specific immunity and acquired immunity. TLR9 agonist (CpG ODN) and TLR3 agonist (poly I:C) are nucleic acids-based adjuvant resembles with DNA or RNA virus and known for the main Th1 polarized immune response modulator.

Collected results have supported the combination of this kind of TLR agonists may offer great advantages in amplifying signals through two different adaptor molecule (MyD88 and TRIF) and improving Th1 responses-biased vaccine efficacy. But still, the underlining mechanism and effect of TLR synergy has remained unclear. The putative discovered mechanism about synergistic effect of TLRs was confined in *in vitro* analysis whereas the adjuvanticity of CpG ODN and poly IC in *in vivo* were presented in a diverse way depends on the combined vaccine and administration routes.

Here, optimal medium for multi-epitope vaccine production was developed and CpG ODN and poly I:C mediated TLR synergy were evaluated to construct efficient culture and adjuvant system for subunit vaccine against FMD.

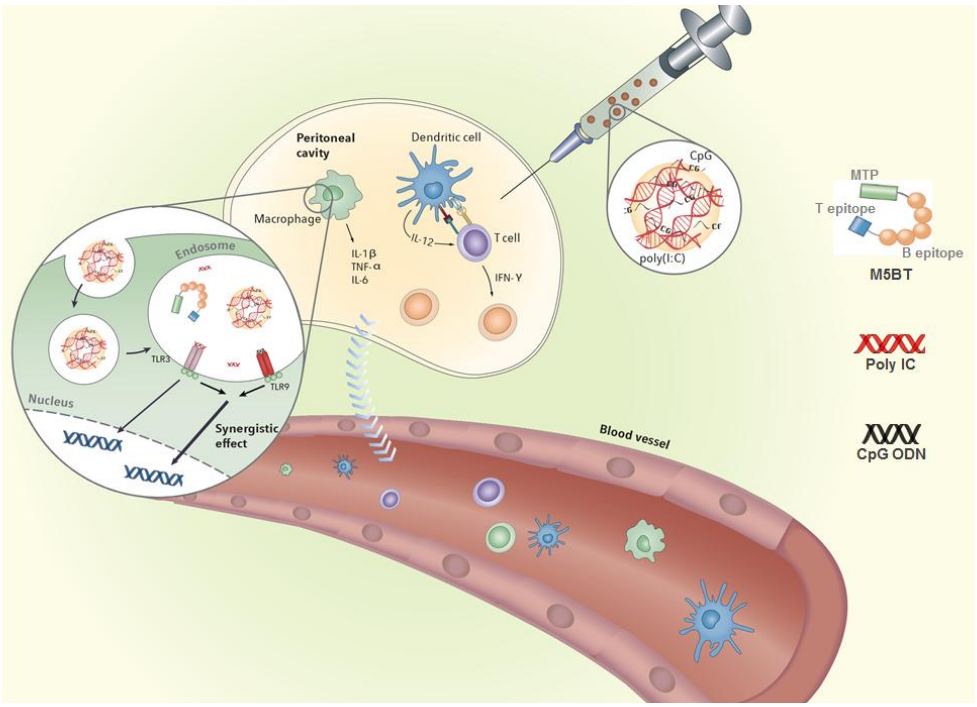

Figure 1. Graphical abstract of the study

II. Review of Literature

1. FMD and vaccine

1) FMD

Foot-and-mouth (FMD) disease is one of the highly contagious livestock disease having effects on *artiodactylae* such as cattle, swine and sheep. The infectious agent, FMD virus (FMDV) transmitted through the respiratory tract, fecal-oral route and direct contact by infected individuals can cause severe lesions displayed as mouth blisters, foot sores, high fever and myocardial fibrosis especially in young cloven-hoofed animals (Grubman & Baxt, 2004). Although the mortality rates of FMD is relatively low (below 5%), still the FMD recurrence led to the enormous economic damages with respect to productivity and trade in livestock industry (Ramirez-Carvajal & Rodriguez, 2015).

In the meanwhile, the geographically distributed FMDV have impacted on not only European countries but also Asia (Table. 1). Especially, FMD outbreak in Korea (2010 - 2011) incurred huge damages to Korean society and hindered the development of livestock industry. The government estimates of total economic losses were around 3-billion dollars and over 3-million animals were slaughtered for prevention of latent viral propagation (Lee et al., 2011).

Although the needs for development of a vaccine against FMDV have increased, high transmission rate, broad host range, and antigenic diversity made hard to control this disease (Longjam et al., 2011; Brito et al., 2015).

Table 1. FMDV distribution areas by serotypes (modified from Zhang et al., 2011)

Area	Main serotypes in FMDV distribution areas						
	Type O	Type A	Type C	Type Asia 1	SAT-1	SAT-2	SAT-3
China	+	+	−	+	−	−	−
Southeast Asia	+	+	−	+	−	−	−
Africa	+	+	−	−	+	+	+
Middle East	+	−	+	+	−	−	−
United Kingdom	+	+	−	−	−	−	−
South America	+	+	+	−	−	−	−
Korea[1]	+	+	−	−	−	−	−

[1] Serotype distribution in Korea was added. '+' means positive, and '−' means negative

2) FMDV

The causative agent, FMDV is ssRNA positive-strand virus with approximately 8.4 Kb of genome size. In a taxonomic aspect, FMDV is classified within the genus *Aphthovirus* of the family *Picornaviridae* including equine rhinovirus and bovine rhinitis A virus (Sáiz et al., 2002). The virions of *Aphthovirus* consist of non-enveloped icosahedral symmetry capsid and in the case of FMDV, as shown in Figure 2, the 60 copies of capsomer comprised of four structural proteins (Exposed VP1, VP2 and VP3 and internally located VP4) construct the capsid structure. In particular, the VP1 encoding sequences have been utilized on FMDV strains characterization and vaccine design referred the elucidated previous experiments that VP1 played important roles in virus entry into susceptible cells, serotype specificity and protective immunity (Fry et al., 1999; Cottam et al., 2008; Mohapatra et al., 2011).

In comparison with the function of structural protein in FMDV, non-structural proteins (NSPs) are involved in viral genome replication (2B, 2C, 3A, 3B and $3D^{pol}$ protein) and protein processing ($3C^{pro}$ protein) (Jamal & Belsham et al., 2013)

Since the first FMDV was found by Loeffler and Frosch in 1897, seven serotypes of FMDV have been sequentially reported: Serotype O, A, C, SAT-1, SAT-2, SAT-3 and Asia-1. Among these, FMDV serotype O was known for the major causative agent of FMD occurred in Korea, 2011 (Rodriguez & Grubman, 2009).

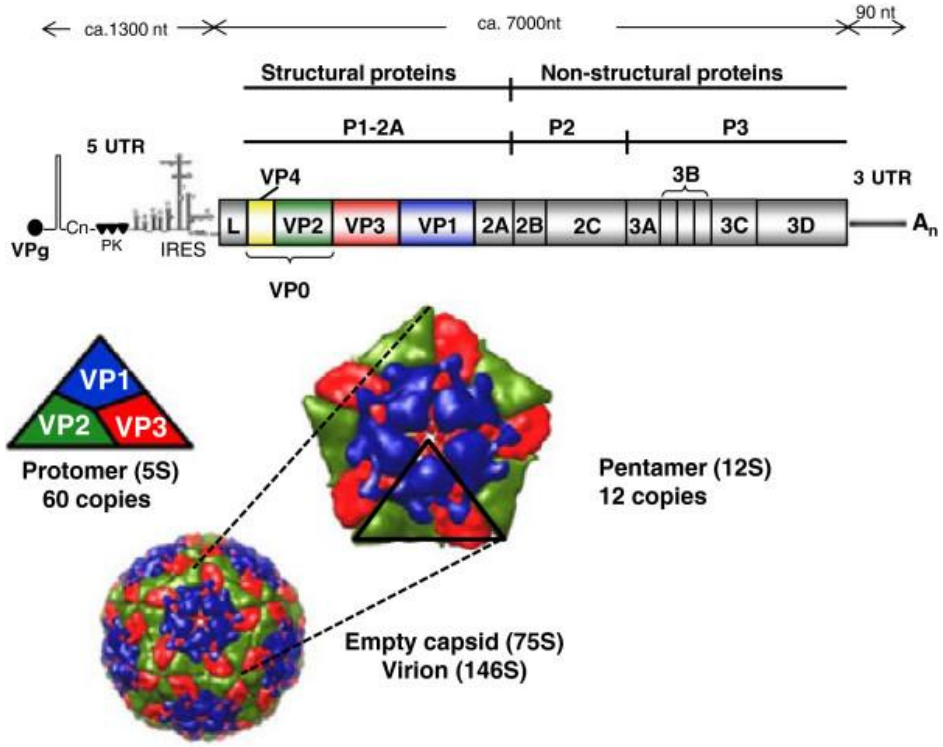

Figure 2. Genome organization and the structure of FMDV (Jamal & Belsham et al., 2013)

3) Vaccine strategies against FMDV

As domestic animals were raised with relatively short-term under the industrial needs, generation of protective immunity by vaccinization had not been the first requirement in livestock industry (Meeusen et al., 2007). However, the recent days have been seen the vaccinization on livestock is crucial strategies to enhance productivity and ensure the food security considering increased needs for animal well-fare and a ban on antibiotics (Casewell et al., 2003).

With the request for the development of the cost-effective vaccine against FMD, experiments have been performed using a whole virus or partial fraction of it. Simply, the vaccine strategies can be categorized into conventional and next-generation vaccine (or new-generation vaccine) as listed in the following Table 2.

Conventional vaccine such as live attenuated vaccine or killed vaccine use whole virus, but not infectious form preceded by attenuation through constitutive adaptation on susceptible baby hamster kidney (BHK) cells (Amadori et al., 1997) or inactivation treated chemical agents like ethyleneimine (Brown et al., 1963). As this types of vaccine have a similar structure with the intact virus, broad and relatively durable immune response required for protection against the virus was achieved in body systemic compartment.

However, despite the advantages of the conventional vaccine, the shortcomings of it were brought up. First, potential hazard like incomplete viral inactivation or reversion of virulence by mutation raised concerns with the biosafety issues. Also, short

shelf-life and cold chain system limited the vaccine production and furthermore, the strategies of live attenuated vaccine production spend long time for adaptation on the foreign host which made it difficult to produce required serotypes or subtypes in time (Rodriguez & Grubman, 2009).

By figuring out the structures of the virus and the key components required for virus infection, the new vaccine strategies could be introduced: DNA vaccine using plasmid encoding a structural protein of FMDV induced protective immune response in murine and swine model (Wong et al., 2000). This strategy had no risk for infection whereas potential insertional mutagenesis, high expense, limited transgene size and tolerance were addressed as the limitation of DNA vaccine (Hacein-Bey-Abina et al., 2003; Fioretti et al., 2010).

Meanwhile, the researches on subunit vaccine for FMD have been reported. Subunit vaccine was also designed as alternatives to conventional vaccine. Basically, this type of vaccine is non-infectious and easily manipulated by genetic engineering such as deletion of oncogenic part of pathogen and combination between antigenic protein encoding sequences (Zhang et al., 2011). Moreover, the subunit vaccine preparation is relatively simple so it does not require cold-chain and allows differentiation of infected from vaccinated animals (DIVA) (Kumru et al., 2014).

Table 2. Vaccine strategies

Vaccine	Features
Conventional vaccine	
Live attenuated vaccine	- Attenuated virus through several passages in foreign host (Egg, cell and animal) - Removed virulence of pathogen via organism to make virus less harmless
Killed vaccine (or Inactivated vaccine)	- Virus grown in culture and subsequentially inactivated with formaldehyde, binary ethyleneimine (BEI), Heat or UV - Adjuvanted with alum or oil emulsion
Next generation vaccine	
DNA vaccine	- Genetically engineered DNA producing antigen using host cell
Subunit vaccine	- Comprised of specific-protein antigens or recombinant protein components
Synthetic vaccine	- Synthetic peptides and carbohydrates as antigens
Virus-like particle (VLP)	- Non-infectious particle comprised of structural proteins like envelope or capsid

4) Multi-epitope vaccine for FMD

One of the newly emerging subunit vaccine strategies is multi-epitope vaccine comprised of various epitopes recognized by B cell and T cell called as B epitope and T epitope, respectively (Bijker et al., 2007). It has seen T epitope is needed to stimulate T lymphocytes (Van der Burg et al., 2006; Brun et al., 2011) and B epitope can induce an antigen-specific humoral immune response by eliciting neutralizing antibodies from plasma cells (Tam & Lu, 1989; Li et al., 2014).

The multiple epitopes (or antigenic determinants) can be synthesized in a chemical or biological way resulted in a various structure such as linear polypeptide or dendrimer (Heegaard et al., 2009). Chemically synthesized dendrimer bearing multi-epitope for FMD showed less sensitivity to degradation whereas difficulties of vaccine production and disturbed individual epitope recognition were addressed (Blanco et al., 2013).

On the contrary, through biological engineering, a specific gene expressing multi-epitope can be incorporated into a host like *E.coli*, *Pichia pastoris* (Zhu et al., 2012), a plant cell (Gil et al., 2001) or animal cell. In addition to the advantages of subunit vaccine, multi-epitope vaccine from bioreactor can be rapidly produced without virus leakage and covers the broad antigenic spectrum of FMD.

It has been shown the multi-epitope vaccine using structural protein, VP1 elicited protective immunity against FMD. Since the VP1 region $_{144-159}$ was firstly discovered as a major epitope (Pfaff et al., 1982), the protruding Arg-Gly-Asp (RGD) motif included in G-H loop of VP1 region $_{140-160}$ was broadly investigated and

figured out this region interacted with cell membrane through integrin αβv6-mediated cell entry (Berryman et al., 2005; Burman et al., 2006). Thus, strategies applying G-H loop for multi-epitope vaccine seem promising due to efficiently induced abntigen-specific antibodies can prevent the FMDV entry from the beginning of FMD infection (Figure 3).

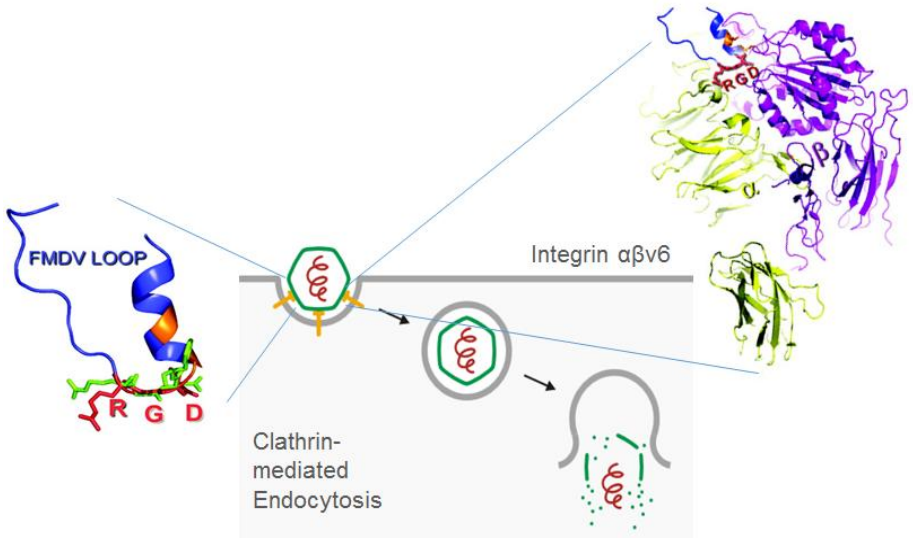

Figure 3. FMDV infection via interaction with integrin αβv6. modified from the Structure-based modeling of the FMDV peptide with integrin αβv3 in Burman et al., 2006.

2. Adjuvant system

1) Vaccine adjuvant

An adjuvant is a substance added to vaccine to restore or improve the immunogenicity to antigen. Since adjuvant was firstly defined as “substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone” (Ramon, 1924), many trials have been done to develop ideal vaccine adjuvant (Aiyer Harini et al, 2013; Awate et al., 2013).

Basically, adjuvant aims to improve immunogenicity in various way by maximizing immune responses to a vaccine, guiding types of antigen-specific adaptive immunity, altering speed of initial immune response or operating memory response (summarized in Table 3). The study related to adjuvant have been done for around 100 years and recently, the number of licensed vaccine with adjuvant has been increased as shown in Figure 4 (Pasquale et al., 2015) even though still their mechanism of action were not well clarified (Figure 5).

Therefore, there have been a lot of efforts in order to assess the understanding about the adjuvant effect and thereby, the types of adjuvant have been classified into carrier adjuvant and immunostimulatory adjuvant based on its characterized mechanism of action (Singh & O’Hagan, 2003).

Table 3. Benefits of adjuvants (modified from Lee & Nguyen, 2015)

Benefits of adjuvants
1) Reduce the antigen dose and number of vaccinization
2) Improve vaccine efficacy in infants, the elderly and immunodeficient patients
3) Increase functional antibody titer
4) Induce rapid and prolonged immune responses
5) Provoke robust cell-mediated immunity
6) Provide broad protection via cross-reactivity
7) Potentiate mucosal immune response

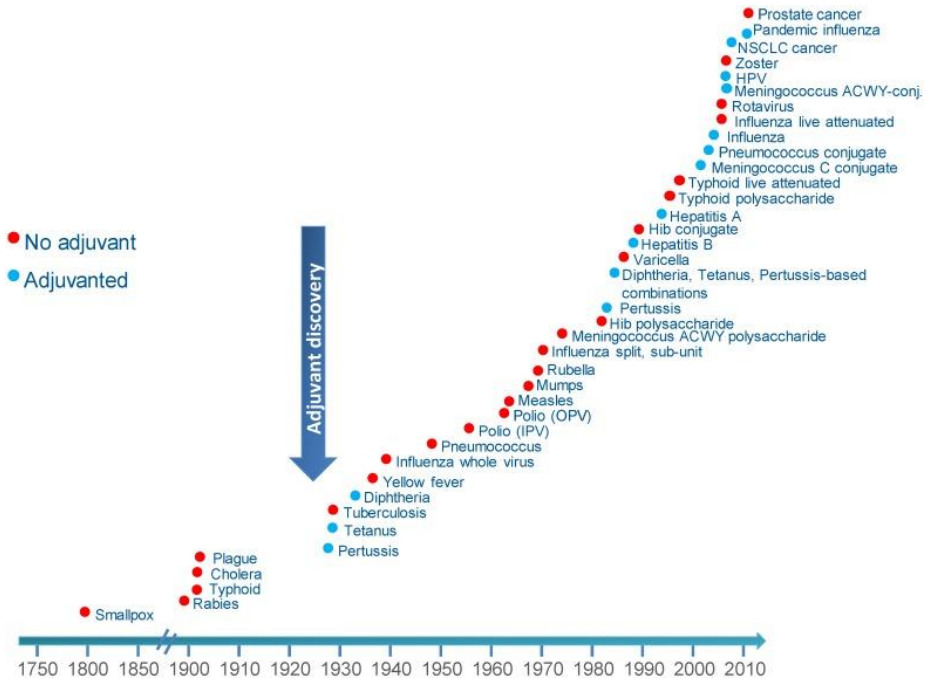

Figure 4. Licensed vaccines with or without adjuvant (Pasquale et al., 2015)

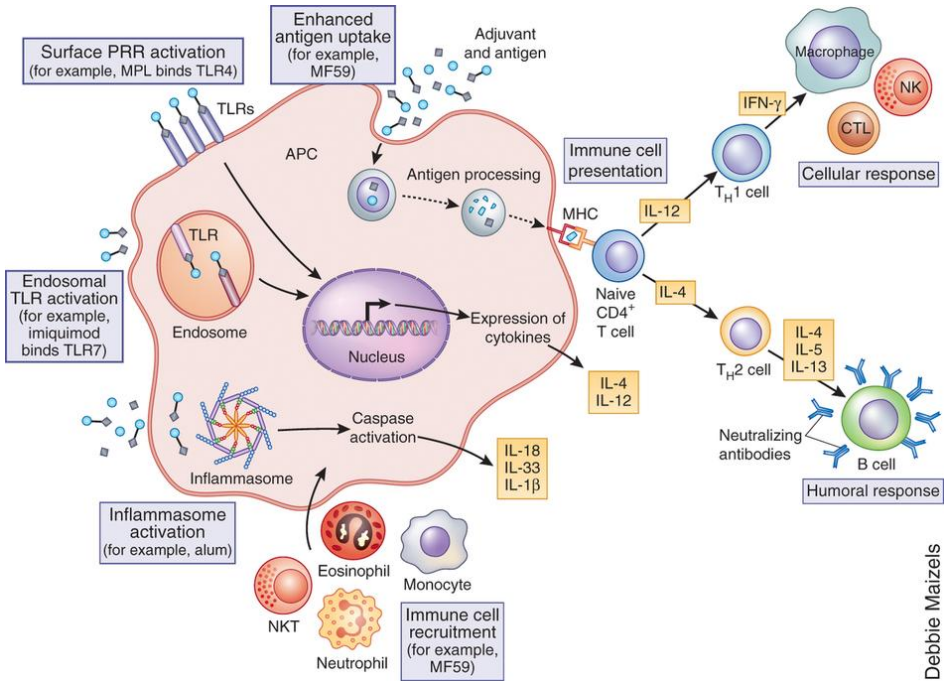

Figure 5. Putative mechanisms of action of adjuvants (Reed et al, 2013)

(1) Carrier adjuvant

The carrier adjuvants (or delivery system) such as aluminium salts, liposome, oil-in-emulsions, polymeric carrier and immune stimulating complexes (ISCOMs) have been combined with vaccines (Marrack et al., 2009).

Aluminium salts are the classic adjuvant widely used in the human vaccine. It was known that slowly released antigen from insoluble particle-elicited prolonged immune response defined as 'depot effect'. The main forces to generate salt particles with protein have been understood by the electrostatic interaction and anionic ligand exchange (Hem & HogenEsch, 2007). Since the adjuvanticity of aluminium salts was demonstrated in terms of antigen uptake and presentation in macrophages (Mannhalter et al., 1985), the experiments using peripheral blood mononuclear cells (PBMCs) indicated aluminium salts elicited up-regulation of MHC class II molecules (CD40 and CD86) (Ulanova et al., 2001). Not only the innate immune response stimulated by aluminium salts, but also antigen-specific adaptive immune responses are reviewed at Figure 6.

Liposomes are comprised of self-assembled phospholipid and cholesterol encapsulating an aqueous core, so they perform like carrier to prevent the vaccine from degradation.

Oil-in-emulsion adjuvants made by bio-degradable squalene oil were commercialized with influenza vaccine named as MF59 and AS03.

Polymer-based adjuvants are categorized: synthetic polymers, nature polymers (chitosan and alginate) and copolymer had a diverse function to potentiate immune response or protect cargo

to the target sites.

ISCOMs are the spherical structure bearing exposed antigen on the surface. The enhanced immune responses by ISCOMs still remain unclear. The elevation of MHC II expression on antigen-presenting cell (APC), cytokine profiles were dependent on the combination of antigen and ISCOMs (Kersten & Crommelin, 2003)

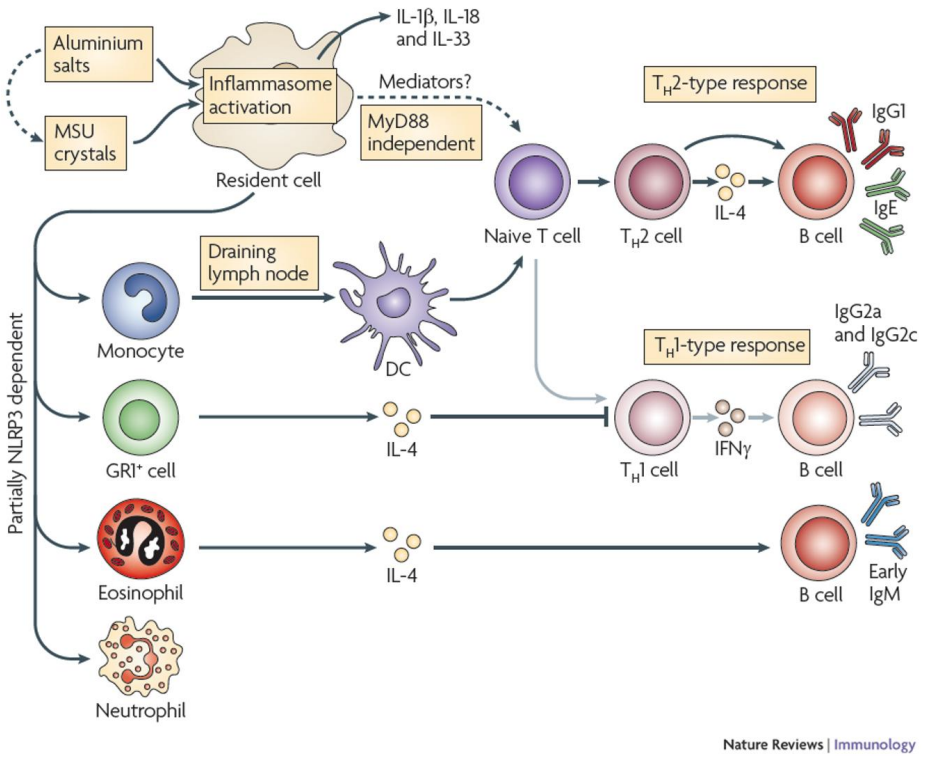

Figure 6. Adaptive immune response induced by aluminium salts (Marrack et al., 2009)

(2) Immunostimulatory adjuvant

Immunostimulatory adjuvants like cytokines and TLR agonists play the major role in the activation of innate immunity and bridge the alerted immune response to antigen-specific immunity (or adaptive immunity).

TLR agonists have been broadly tested because its diversity and complexity have potential to provide properly amplified or guided immune response depends on the combination with the vaccine (Lee & Nguyen, 2015).

IFN-ɣ and granulocyte-macrophage colony stimulating factor (GM-CSF) are representative pleiotropic cytokines. These cytokine adjuvants activated primary immune response and contributed to enhanced cellular immune responses. The recent application of cytokine adjuvants has been found in DNA vaccines strategy (Petrovsky & Aguilar, 2004).

The crucial issue in the adjuvant application is toxicity, even though various adjuvants have been introduced, high toxicity exhibited in clinical trials limited its uses. The following Table 4. listed the carrier and immunostimulatory adjuvants for research and clinical phases.

Table 4. Clinically used and tested adjuvants (modified from Reed et al., 2013)

Adjuvant name	Class	Mechanism or receptor	Type of immune response	Clinical phase or licensed product name
Carrier adjuvants				
Aluminum salts e.g. aluminumoxyhydroxide aluminumphosphate	PF	Nalp3, ITAM, Ag delivery	Ab, Th2	Numerous licensed products
Virosomes	PF	Ag delivery	Ab, Th1,Th2	Epaxal, Inflexal V
Oil-in-emulsions e.g.MF59,AS03,AF03,SE	PF	Immune cell recruitment, ASC, Ag uptake	Ab, Th1, Th2	Fluad, Pandemrix
ISCOMs e.g. saponin, phospholipid	C	Unknown	Ab, Th1,Th2, CD8+ T cells	Phase 2
Immunostimulatory adjuvants				
dsRNA analogues e.g.polyI:C	IM	TLR3	Ab, Th1,CD8+ T cells	Phase 1
Lipid A analogues e.g.MPL, RC529, GLA, E6020	IM	TLR4	Ab, Th1	Cervarix, Supervax, Pollinex Quattro, Melacine
Flagellin	IM	TLR5	Ab, Th1, Th2	Phase 1
Imidazoquinolines e.g.Imiquimod, R848	IM	TLR7 and TLR8	Ab, Th1	Aldara
CpG ODN	IM	TLR9	Ab, Th1, CD8+ T cells	Phase 3
Mixed adjuvants				
AS01 (MPL,QS21, liposomes)	C	TLR4	Ab, Th1, CD8+ T cells	Phase 3
AS02 (MPL,QS21, emulsion)	C	TLR4	Ab, Th1	Phase 3

Ab, antibody; Ag, antigen; ASC, apoptosis-associated speck-like protein containing caspase recruitment domain; C, combination; IM, immunomodulatory molecule; ITAM, immunoreceptor tyrosine-based activation motif; PF, particulate formulation; TDB, trehalose dibehenate.

3. TLR agonist

1) TLR

The family of pattern-recognition receptors (PRRs), toll-like receptors (TLRs) were conserved receptors named after *Toll* in *Drosophila melanogaster*. TLRs recognize a wide variety of pathogen-associated molecular patterns (PAMP, e.g. nucleic acid, lipid, carbohydrate and peptide) from bacteria, viruses and parasites (Table 5).

The location of TLRs has been classified and characterized according to the recognized TLR agonists (or ligands). TLRs expressed on the surface or on the endocytic vesicle membrane consist of ectodomain for ligand binding, transmembrane domain and cytoplasmic domain for a signal pathway through numerous transcription factors (Botos et al., 2011).

Basically, TLRs on the cell surface (TLR1, 2, 4, 5 and 6) induce Th2 type responses. On the contrary, TLR3, 7/8 and 9 located within endoplasmic reticulum and rapidly recruited to endosomal-lysosomal compartments provoke Th1 type responses. TLRs, TLR agonists and subsequential signal cascades for cytokine production were depicted in Figure 7.

It has been understood the TLRs sense infection and initiate the innate immune response. Recently, not only non-specific immune cell activation and inflammation but also adaptive immune responses through the interaction between T cell and activated dendritic cells (DCs) have been recognized as the crucial effect of TLRs (Bhardwaj et al., 2010). The reported effects of TLR agonists on up-regulation of MHC class II and co-stimulatory signal molecule and increased inflammatory

cytokine release by DCs sequentially led to enhanced antigen uptake and presentation of activated DCs.

Table 5. Pathogens expressing multiple TLR ligands (modified from Trinchieri & Sher, 2007)

Pathogen	TLR	TLR ligand
Mycobacterium tuberculosis	TLR2	Lipoarabinomannan
	TLR4	Phosphatidylinositol mannosides
	TLR9	DNA
Salmonella typhimurium	TLR2	Bacterial lipoprotein
	TLR4	Lipopolysaccharide
	TLR5	Flagellin
Haemophilus influenzae	TLR2	Lipoprotein
	TLR4	Lipopolysaccharide
Candida albicans	TLR2	Phospholipomannan
	TLR4	Mannan
	TLR9	DNA
Murine cytomegalovirus	TLR2	Viral protein
	TLR3	Double-stranded RNA
	TLR9	DNA
Herpes simplex virus	TLR2	Viral protein
	TLR3	Double-stranded RNA
	TLR9	DNA
Toxoplasma gondii	TLR2	Glycosylphosphatidylinositol anchor
	TLR11	Profilin

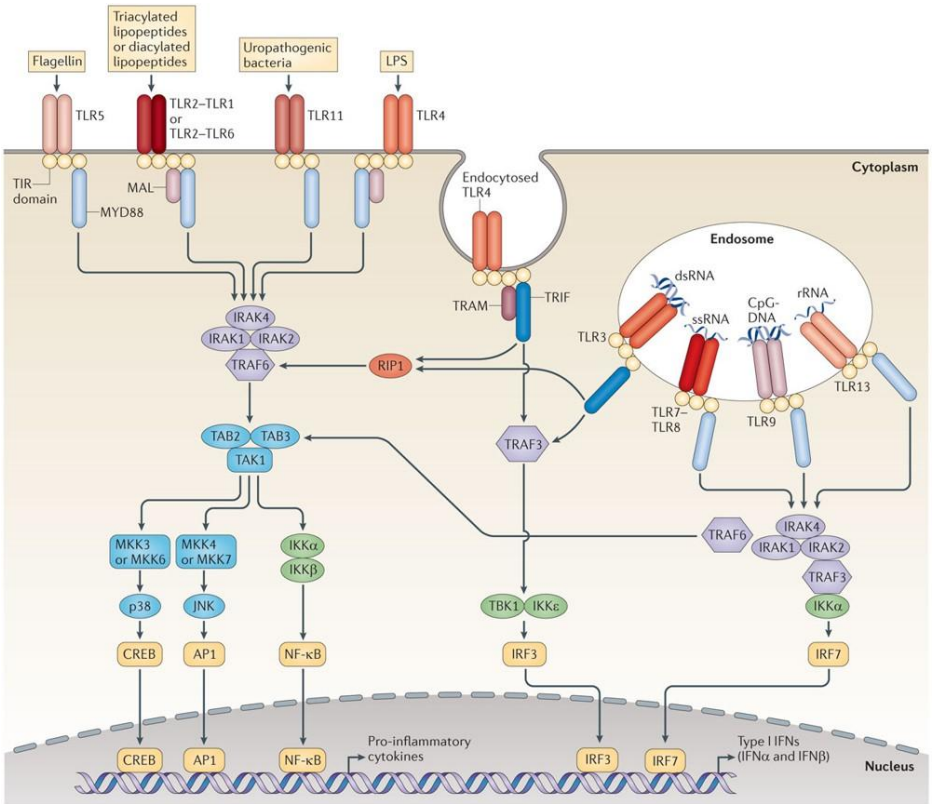

Figure 7. TLR signaling pathway (O'Neill et al., 2013). lipoprotein and lipoteichoic acid (LTA) are recognized by TLR2 combined with TLR1 and TLR6, respectively. TLR4 recognizes lipopolysaccharide (LPS) and Monophosphoryl lipid A (MPL). Flagellin was recognized by TLR5; TLR3, 7/8 and 9 can detect microbial nucleic acids (dsRNA, ssRNA and unmethylated CpG motifs of ssDNA, respectively).

2) CpG ODN

A short synthetic ssDNA containing unmethylated cytosine triphosphate deoxynucleotide followed by guanine triphosphate deoxynucleotide (CpG motif) is called CpG ODN (or CpG DNA). TLR9 expressed in B cells and plasmacytoid DC (pDC) is known to recognize CpG motifs. As listed in Table 6. CpG-ODNs have been classified into three classes: A-, B- or C-Class ODNs based on modification in backbones (phosphodiester bond, PO or phosphorothioate bond, PS), location and number of CpG motifs, and palindrome (Klinman, 2004).

The constitutive approaches on CpG ODN have investigated to disclose the mechanism of recognition between TLR9 and CpG ODN. It was seen phosphoinositide 3-kinase (P13-kinase) played a important role in CpG ODN shuttling to TLR9 in Ishii's experiments (Ishii et al, 2002) whereas the results from Latz indicated CpG ODN entered Rab5-positive-early endosome through clathrin-dependent but caveolin-independent manner and then, recognized by TLR9 which is normally localized in endoplasmic reticulum (ER) but redistributed to endosome upon stimulation (Latz, et al., 2004).

As widely reviewed at Figure 8, The immunomodulatory effects of CpG ODN have distinct characteristic according to its classes. Especially, A-Class CpG ODN activates pDCs and natural killer cells (NK cells) but induces little B cells activation leading to cellular adaptive immune response represented as Th1 cell and cytotoxic T lymphocytes (CTLs) and humoral (B cells) immune responses (Ishii & Akira, 2006).

On the other hand, B-Class CpG ODN provokes polarized B cells activation in terms of its proliferation and differentiation rather than pDCs. In recent, IFN-producing killer dendritic cells (IKDCs) were also known to be activated by B-Class CpG ODN. In the case of mice, A- and B-Class CpG both activate conventional DC (cDC).

Table 6. Classes of CpG ODN

ODN class	Example ODN	Structural features	Immune effects
A-Class (or D-type) for IFN-α inducing	GGgggacgatcgtcgGGGGG	- Mixed PO and PS backbone - The center portion with the CpG motif(s) - CpG flanking region forms a palindrome - Poly-G tail at 3' end	Induce strong pDC IFN-α secretion moderate expression of co-stimulatory molecules Induces very little B cell activation
B-Class (or K-type) for B cell activating	TCGTCGTTTTGTCGTTTTGTCGTT	- Fully-PS-modified backbone - Multiple CpG motifs - No major secondary structure - CpG motif for hTLR9 activation is at the 5' end	Induces very strong B cell proliferation, differentiation Induces modest IFN-α secretion
C-Class (for combined activities)	TCGTCGTTTTCGGCGCGCGCCG	- Fully-PS-modified backbone - 1 or more CpG motifs - Self-complementary palindrome - form duplex or hairpin structure	Induces strong B cell proliferation, differentiation Induce strong pDC IFN-α secretion

Capital letters, PS bond; lower-case letters PO bond; underlines, self-complementary palindromes; bold letters, CpG motifs thought to confer immune stimulation.

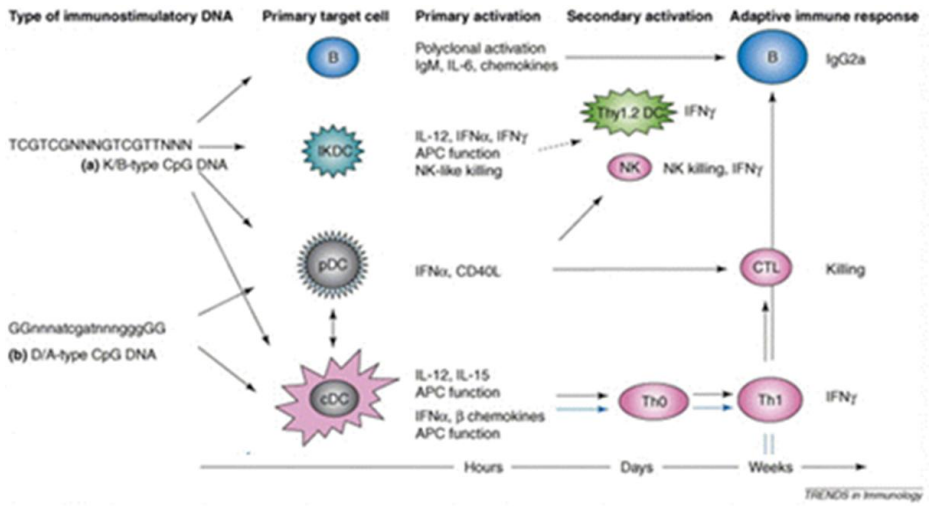

Figure 8. Innate and adaptive immune modulation by different class CpG ODN slightly modified from Ishii & Akira, 2006. (A) B-Class (or B-type) CpG ODN and (B) A-Class (or A-type) CpG ODN induced immune response are listed.

3) poly I:C

poly I:C is one of the immunostimulant molecule known to interact with TLR3 expressed on B cells, macrophages and DCs. This synthetic TLR3 agonist is structurally similar to dsRNA presented in some viruses.

Lately, further researches on recognition of poly I:C have elucidated retinoic-acid-inducible gene-I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5) are also involved in dsRNA sensing in addition to TLR3 shown in Figure 9, These two proteins shared caspase recruitment domain-RNA (CARD-RNA) helicase and thereby recognized viral dsRNA induced antiviral response (Meylan et al., 2006)

The adjuvanticity of poly I:C have been reported. Direct activation of DCs and control of NK cells to kill tumor cells were confirmed (Sivori et al., 2004). In an aspect of adaptive immune responses, poly I:C provoked prolonged survival of tumor-bearing rodents or improved antigen-specific $CD4^+$ T cells or $CD8^+$ T cell responses, particularly in the presence of anti-CD40 stimulation (Soares et al., 2007).

Taken together, these results have implied the potency of poly I:C in clinical uses (Martins et al., 2015). Several approaches have tried to use poly I:C as vaccine adjuvant with modification aimed to enhance immunogenicity but decrease toxicity (depicted at Figure 10).

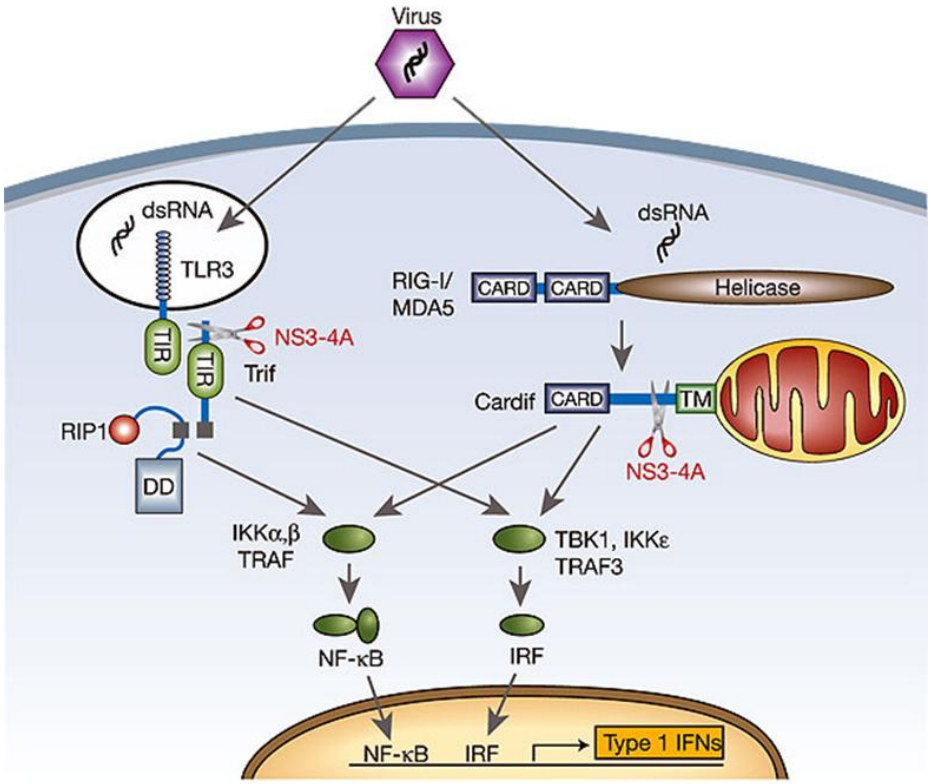

Figure 9. Recognition of dsRNA from virus by receptors, TLR3 and RIG-I/MDA5 (Meylan et al,. 2006)

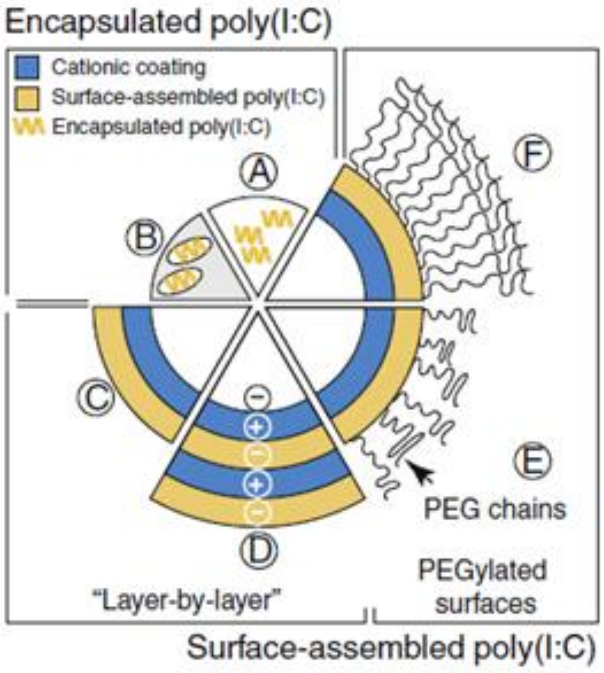

Figure 10. Schematic architectures of microsphere formulations for poly I:C delivery (Hafner et al., 2013). A - B: Encapsulation of poly I:C. C - F: Surface-assembly of poly I:C using polymer such as poly Lactic-co-Glycolic Acid (PLGA), polyethylene glycol (PEG), polylysine (PLL) and diethylaminoethyl (DEAE)-dextran.

4) TLR synergy

Collected studies on TLRs drew a new emerging phenomenon called as TLR synergy. At an earlier time, synergy was defined as increased cytokine production by simultaneous stimulation of certain TLR agonists combinations rather than an individual. The major cause of the improved cytokine signals was derived from different signaling pathways activated by receptors using two adaptor molecules, Myeloid differentiation primary-response gene 88 (MyD88) and TIR-domain-containing adaptor protein inducing IFN-β (TRIF) (Figure 11). In recent, numerous approaches about TLR synergy elucidated the synergistic effect of TLR are not limited in cytokine production but also modulate immune cells.

Particularly, synergistic effects of TLR3 and TLR9 have been evaluated *in vitro* and *in vivo* based on 'MyD88-TRIF adaptor cross-talk' (Tan et al., 2014). Up-regulation of IL-1α, IL-6, IL-33 were confirmed on murine macrophage cell line by microarray (Tross et al., 2009) and increased IL-12 from the dendritic cell was also determined (Krummen et al., 2010). Interferon regulatory factor 5 (IRF5) mediated synergy between TLR3 and TLR9 was claimed by Ouyang but due to complexity of TLR mechanism, still left things to be understood (Ouyang et al., 2007).

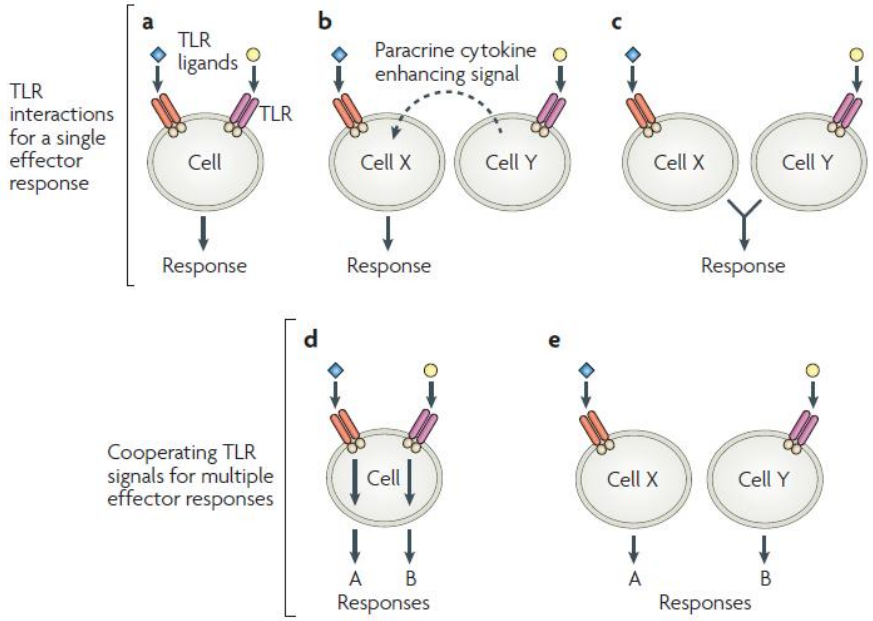

Figure 11. TLR cooperation mechanism for the host defense against infection. Effector response in (a-e) induced by individual or multiple TLR signals on a single or different cell types in autocrine or paracrine manners of cytokine signals in order to mediate host resistance (Trinchieri & Sher, 2007).

III. Materials and Methods

1. Preparation of protein

1) Strain, plasmid and protein

The strain *E.coli* BL21 (DE3) contained an artificial protein (M5BT)-expressing pET21a was used in order to study the effects of microbial growth according to the medium composition. As a candidate for multi-epitope vaccine for FMD, M5BT was designed to have 5 B epitope, T epitope and M cell targeting moiety. The protein was expressed from recombinant *E.coli* under the control of the *Lac* operon induced by IPTG (Calbiochem, USA). Table 7. summarizes the properties of M5BT used in this work.

Prior to the study, the sequences of M5BT was confirmed aligned with the reference sequence from National instrumentation center for environmental management (NICEM) (Figure 12).

Table 7. Physiochemical properties of M5BT

Properties	
Molecular weight (Da)	19130.6
Number of amino acids	183
Theoretical pI	10.07
Total number of negatively charged residues (Asp, Glu)	10
Total number of positively charged residues (Arg, Lys)	20

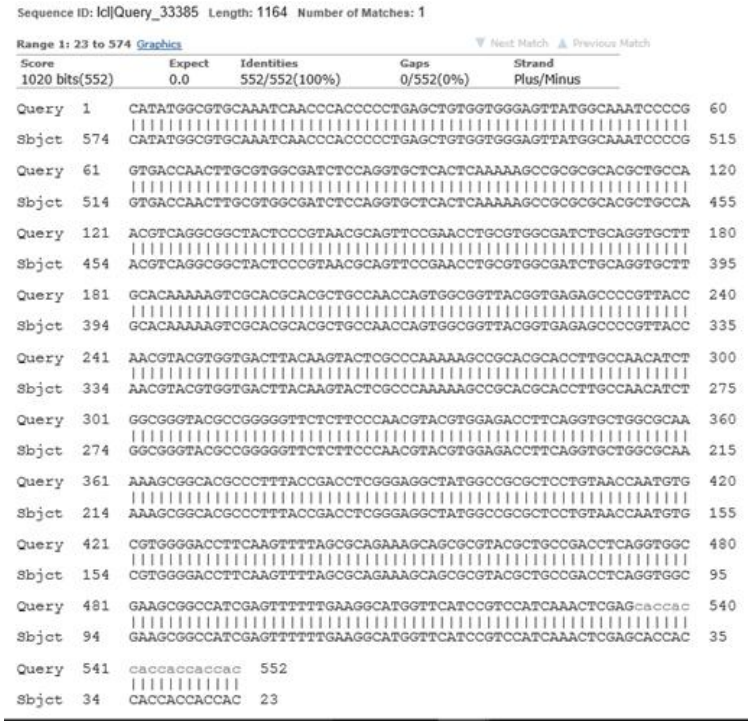

Figure 12. Sequence alignment of plasmid from *E.coli* against references

2) Medium used for *E.coli* growth

Four different media were used for the cultivations; 1) LB (Lysogeny broth); 2) TB (Terrific broth); 3) T1B; and 4) T2B. Conventional LB medium (Sigma, USA) was purchased and TB, T1B and T2B was prepared according to Table 8 using yeast extract and tryptone (Difco, USA), glycerol (LPS solution, Korea), K_2HPO_4, KH_2PO_4 and Lactose (Sigma, USA) and NaCl (Biosesang, Korea). The initial pH of each medium was around 7 without adjustment following by the sterilization at 121 ℃ for 15 min.

For growth measurement of *E.coli*, 50 ml of each medium in 250 ml baffled flasks was inoculated with 0.1% of Ampicillin and 1% of pre-grown *E. coli* culture shaken overnight at 230 rpm and incubated at 37 ℃.

For weight measurement of the target protein, overnight *E.coli* culture was diluted in fresh medium (400 ml in 2 L baffled flask) with the addition of 0.1% Ampicillin. 0.5 mM IPTG for LB and 1mM IPTG for TB, T1B and T2B were used for induction at specific $O.D_{600}$ (about 0.5 $O.D_{600}$ for LB and over 3 $O.D_{600}$ for TB, T1B and T2B). After 4 hours of incubation, each culture medium was harvested by centrifugation at 6,000 rpm for 10 min and washed twice with cold 1X PBS (HyClone, USA) and thereby, stored in pelletized form at －70 ℃ until use.

Table 8. Composition of media used for shaken flask culture

	LB	TB	T1B	T2B
pH	7.41	7.34	6.8	7.43
Component (l^{-1})				
Yeast extract (g)	5	24	24	24
Tryptone (g)	10	12	12	12
Glycerol (ml)		8	4	4
K_2HPO_4 (g)		9.4	12	12.5
KH_2PO_4 (g)		2.2	3	2.3
NaCl (g)	10		5	
$MgSO_4$ (Fin. mM)			2	10
Lactose (g)			5	

(1) Growth curve

To obtain cell density at each time point, $O.D_{600}$ was measured with Eppendorf BioPhotometer (Eppendorf, Germany). Samples were diluted to maintain the ideal O.D range between 0.01 and 0.8.

(2) Relative expression of M5BT

The relative expression ratio of M5BT was determined by SDS-PAGE and subsequent coomassie staining. Before the analysis, 10 ml of 1X PBS was added to the wet cell pellets for sonication comprised of a pulse on for 5s and pulse off for 2s for 2 min. Only 1 ml of resuspended solution was used for analysis.

The soluble fraction of M5BT was acquired by centrifugation of sonicated solution at 17,000 rpm for 20 min at 4 ℃. Then, 10 mM Tris-NaOH (pH 12.5) was added to the pellet to collect an insoluble fraction of M5BT, followed by repeated centrifugation.

Supernatant of each step was collected for SDS-PAGE (Stacking at 70 V for 30 min, Resolving at 140 V for 70 min). In this case, TB, T1B and T2B were 4-fold diluted to analyze the intensity of protein band between the proper range and thereby, the relative band intensity from the different medium was displayed and quantified by Image Lab statistical software (Bio-Rad, USA).

3) Ni-NTA affinity chromatography

The wet pellets were dissolved in 20 ml of binding buffer with 0.1% of lysozyme and kept on ice prior to sonication (10 s/pulse on; 5 s/pulse off for 8min). Next, the clear supernatant was

acquired by centrifuged disrupted lysate at 17,000 rpm for 15 min at 4℃ and filtrated by 0.45 um syringe filter (Sigma, USA) to exclude debris.

The composition of various buffers used for Ni-NTA affinity chromatography was shown in Table 9. The process for purification followed the general protocol using Ni-NTA affinity chromatography with some modification (Crowe et al., 1994). Briefly, His-bind resin (Novagen, USA; capacity 8 mg/ml; 4 ml for LB, 6 ml for TB and T2B were used) was packed into a column with the same volume of binding buffer and then, washed the column with 5 volumes of binding buffer. Add 5 volumes of charging buffer and subsequently equilibrated with 10 volumes of binding buffer to remove uncharged nickel ion. After the filtrated solution was slowly (flow rate = 1-2 mg/min) loaded onto the resin, washing buffer 1 and washing buffer 2 were sequentially added to remove non-specific protein interacted with Ni-NTA. polyhistidine-tagged M5BT was eluted with 20 ml of elution buffer. Each fraction (SM, SFT, W1, W2 and EF) was collected to evaluate the purification efficiency of M5BT by SDS-PAGE.

Next, eluted protein was dialyzed to remove salts ahead lyophilization. It was contained in tighten membrane (Spectrum labs, USA) and stirred in 5 L of distilled water at 4℃ chamber for 24 h by changing water three times.

Table 9. Buffer compositions for Ni-NTA affinity chromatography

	Binding buffer	Charging buffer	Washing buffer		Elution buffer	Strip buffer
			1	2		
Imidazole (mM)	5	-	5	40	1000	-
Tris-Cl (mM)	20	-	20	20	20	20
NaCl (M)	0.5	-	0.5	0.5	0.5	0.5
$NiSO_4$ (mM)	-	100				
EDTA	-	-	-	-	-	100
pH	7.9	-	7.9			
Volume[1]	5	5	20	10	20ml[2]	3
Flow rate (mg/min)	7-8	6	1-2	1-2	1-2	6

[1]Volume = resin volume

[2]Elution buffer was used at same amount for each medium

4) Quantification of M5BT

The lyophilized M5BT was dissolved in the desired solvent like 1X PBS or distilled water and then the total amount of M5BT was determined by extinction coefficients method (Pace et al., 1995) as following

$$C = (OD_{280} \times \text{mol.wt.}) / (\varepsilon_{280} \times l)$$

ε_{280}, the molar absorbance coefficient ($M^{-1}cm^{-1}$) calculated as $(5500 \times n_{trp}) + (1490 \times n_{trp}) + (125 \times n_{s-s})$; *l*, path-length (cm); *c*, concentration of solute (M); and $O.D_{280}$, absorbance at wavelength 280 nm.

5) Endotoxin removal

To remove endotoxin from M5BT, Endotoxin Thermo Scientific Pierce High-Capacity Endotoxin Removal Spin Column kit (Thermo, USA) was used following the vendor's instructions. Briefly, 3.5 ml of 0.2 M NaOH in 95% EtOH was loaded onto resin included a column for 2 h to regenerate the resin. 3.5ml of 2M NaCl, endotoxin-free water and endotoxin-free buffer (for three times) were sequentially loaded and removed by centrifugation at 500 g for 1 min at 4℃ between every step. Next, 4 ml of purified M5BT solution was slowly applied at a flow rate 10 ml/h for 1 h at room temperature. After new tube was applied to the column, endotoxin removed M5BT solution was acquired after the centrifugation at 500 g for 1 min at 4℃

2. *In vitro* validation of TLR synergy

1) Cell line

The mouse macrophage cell line (Raw264.7; ATCC T1B 71) were cultured in high glucose DMEM (HyClone, USA) supplemented with 10% (v/v) FBS (genDEPOT, USA) and 1% penicillin (100 units/mL) and streptomycin (100 mg/mL). The mouse immuature dendritic cell line (JAWS II; ATCC CRL 11904) were cultured in αMEM (HyClone, USA) including 20% (v/v) FBS, 100 mM sodium pyruvate (HyClone, USA), 5 ng/ml of rmGM-CSF (Peprotech, USA) and 1% penicillin (100 units/mL) and streptomycin (100 mg/mL). The cell line was maintained at 37℃ in a 5% CO_2 humidified incubator.

2) TLR agonists and reagents

CpG ODN was synthesized by Bioneer (Korea) with customized phosphorothiolate-modification (PS) to give a resistance against nuclease. and kept in endotoxin-free water (HyClone, USA). The sequences of CpG ODN used was 5'-ggGGTCAACGTTGAgggggg-3' (PS modified sequences marked as small letters). poly I:C (Sigma, USA) was purchased and dissolved in 0.1% DEPC (Sigma, USA) treated water to prevent degradation. Both TLR agonists were preserved in aliquots at -20 ℃ until use. Previous *in vitro* TLR agonists validation on macrophage cell line was referred with regard to ratio determination (Whitmore et al., 2004) and the concentration of CpG ODN and poly I:C used for *in vitro* study was 0.3125, 0.625, 1.25, 2.5, 5, 10, 20 ug/ml for CpG ODN and 3.125, 6.25, 12.5, 25, 50, 100 ug/ml for poly I:C respectively.

3) *In vitro* stability

CpG ODN and poly I:C were incubated in a stock solution (250 ng/ul) and medium (50 ug/ul in DMEM) at 37℃. After collecting partial fraction at 0, 1, 6 and 24 h, the integrity of CpG ODN and poly I:C was assessed through analysis on gel electrophoresis using 3% TAE and 2% TBE agarose gel with 100 bp and 1 kb DNA ladder (Enzynomics, Korea), respectively.

4) Cell viability

The cytotoxicity effects of TLR agonists on Raw264.7 cell line was analyzed by quantifying the amount of insoluble formazan from mitochondria in viable cells. CpG ODN and poly I:C were treated for 24 hours at 50% of confluent Raw264.7 cell in 96-well plates. Next, 20 ul of MTT reagent (Sigma, USA) were added to each medium-removed well for 4 h to make sure reduction of MTT into formazan crystals, followed by addition of DMSO (Sigma, USA) to solubilize the crystals. The colorimetric detection was done at 570 nm and normalized by the non-treated cell.

5) mRNA expression

(1) Primer design

Macrophage is the prominent cell types involved in innate immune response by producing diverse cytokine signal to activate themselves and other immune cells against pathogens. mRNA expression of pro-inflammatory and anti-inflammatory cyokines was assessed on mouse macrophage Raw264.7 cell line through qRT-PCR technique to confirm whether the combinational stimulation of CpG ODN and poly I:C can induce enhanced immunogenicity *in vitro*. The followings are the primer sequences synthesized by Bioneer (Korea) used for analysis (Table 10).

Table 10. Sequences of real-time PCR primers

Gene		Primer sequence (5' → 3')	T_m (℃)	GC (%)
Il-1β	F	GCCTTGGGCCTCAAAGGAAAGAATC	63.1	52
	R	GGAAGACACAGATTCCATGGTGAAG	61.9	48
Il-6	F	TGGAGTCACAGAAGGAGTGGCTAAG	64.7	52
	R	TCTGACCACAGTGAGGAATGTCCAC	65.6	52
Il-10	F	GGTTGCCAAGCCTTATCGGA	60.9	55
	R	TGCTCCACTGCCTTGCTCTT	63.2	55
Tnf-a	F	GGCAGGTCTACTTTGGAGTCATTG	61.8	50
	R	ACATTCGAGGCTCCAGTGAATTCGG	65.4	52
Gapdh	F	TTCACCACCATGGAGAAGGC	61.1	55
	R	GGCATGGACTGTGGTCATGA	62.5	55
Actb	F	GGCATGGACTGTGGTCATGA	64	48
	R	TAAAACGCAGCTCAGTAACAGTCCG	63.3	48

(2) Preparation of RNA and qRT-PCR

Pre-grown Raw264.7 cells at 50% of confluence in 6-well were individually or simultaneously stimulated with 2.5 ug/ml of CpG and 25 ug/ml of pIC for 24h. RNA preparation was done as described in the manual using TRizol (MRC, USA) reagent. Briefly, Cells were lysed using TRizol followed by addition of chloroform to extrude cytoplasmic RNA by centrifugation at 13,000 rpm for 15 min at 4 ℃. To concentrate RNA, isopropanol was added to RNA solution and incubated at -20 ℃ overnight. Then, pelletized RNA was washed by 70% EtOH dissolved in DEPC treated water and subsequential centrifugation was done at 15,000 rpm for 30 min at a constant 4 ℃. The amount of RNA was quantified using a Nano-Drop spectrophotometer (IMPLEN, Germany).

1 ug of isolated RNA was converted to cDNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover Kit (Toyobo, Japan) following provided manufacturer's procedure. PCR were performed with cycling conditions of 15 min at 37 ℃, 5 min at 50 ℃, 5 min at 98 ℃ using PCR machine T100 Thermal Cycler (Bio-Rad, USA)

Newly synthesized cDNA was mixed with cytokine-specific primers and the SYBR master mix (Enzynomics, Korea). 45 cycles of PCR under the conditions comprised of 7 min at 95 ℃, 30 s at 95 ℃, 30 s at 60 to 63 ℃ depends on the T_m of primers, 60 s at 72 ℃ was carried out using Rotor-Gene Q (QIAGEN, Germany). Relative expression of mRNA was quantified applying comparative Ct calculation. All data were normalized as compared with the quantity of data came from housekeeping RNA input,

GAPDH and ACTB were used in this study. In addition, obtained Ct was once more corrected with the change against Ct of control, untreated sample.

6) BMDC generation

(1) Mice and reagents

6 weeks female C57BL/6 mice (Samtako, Korea) were used corresponding the policy and regulations for the care and use of laboratory animals (Laboratory Animal Center, Seoul National University, Korea). RPMI supplemented with 2% FBS was used for washing during the bone marrow isolation and 10% FBS, 1% penicillin/streptomycin and 50 uM 2-ME (2-mercapto-ethanol) was used for maintenance of the culture condition. Additionally, rmGM-CSF was added at 0, 3 days to differentiate isolated cells into dendritic cells .RPMI 1640 (HyClone, USA), 2-ME and ACK lysis buffer (Gibco, USA) were purchased, respectively.

(2) Isolation of BM cell and culture

Isolation of bone marrow cell was carried out based on the previous study (Jung et al., 2015) with some modifications. Briefly, to expose mouse femurs and tibiae, muscle tissue around bones was removed by scissors and tweezers. Preceded by cutting bones, bones were washed in 70% EtOH for 1min and transferred to the sterile condition. each epiphysis (end of the bone) were flushed out with 2% RPMI in 50 ml falcon tube. After lysis of red blood cells with 1 ml of ACK lysis buffer kept on ice for 1 min, cells are collected by centrifugation at 1,200 rpm for 8 min at 4 ℃.

3×10^6 cells were seeded on a 6-well plate containing 10% RPMI with supplements. The medium was exchanged fresh one added with rmGM-CSF (20 ng/ml) at every third day . On the sixth day of culture, only non-adherent cells were harvested for phenotypic analysis of BMDC.

(3) Validation of BMDC

To determine the phenotype features of dendritic cell. flow cytometry was performed using anti-CD11b conjugated with eFlour 450 and anti-CD11c conjugated with APC (BD Pharmigen, Germany). For analysis, BMDCs were stained with antibodies in 1.5 ml Eppendorf tube washing with FACS buffer (1X PBS with 10% FBS) at constant 4℃. resuspended cells were analyzed on FACS Aria II (BD Bioscience, USA). The cells were regarded as BMDC as The percentage of $CD11b^{-}CD11^{+}$ cells exceeded 65% among the analyzed population (Figure 13).

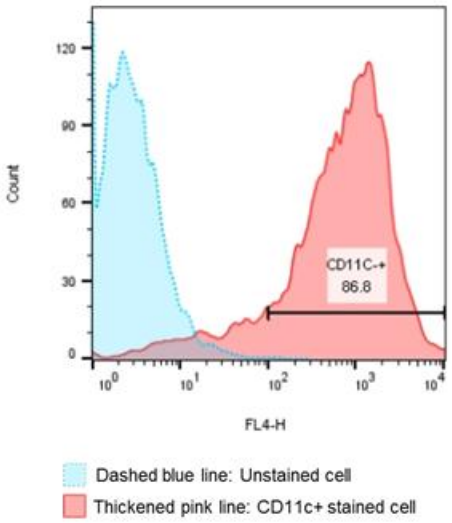

Figure 13. Phenotype characterization of immature BMDC

(4) TLR expression

The sequences of specific primers and its annealing temperatures were designed to determine the expression of TLR in Raw264.7, JAWS II and BMDC, respectively as listed in Table 11.

Isolation of RNA from each cultured cell line and conversion into cDNA were done referred to the procedure used for mRNA expression as previously described.

For PCR, 20 ul of PCR mixture was comprised of cDNA, specific primers and 2X PCR Master mix Solution (*i*-MAX II) (iNtRON, Korea). 30 cycles of PCR was performed comprised of 4 min at 94 ℃, 30 s at 94 ℃, 30 s at 57 ℃, 40 s at 72 ℃. At the end of PCR, TLR expression loaded onto 2% TAE agarose gel was confirmed by gel electrophoresis and visualized using Image Lab statistical software (Figure 14)

Table 11. Sequences of PCR primers for TLR expression

Gene		Primer sequence (5' → 3')	T_m (℃)	PCR products (bp)
Tlr3	F	CAAATCCACTTAAAGAGTTCTCCCCG	60.6	521
	R	CACCAATCCCGTGAAGGTATTGCTTT	63.2	
Tlr9	F	CTAGACGTGAGAAGCAACCCTCTG	63.6	408
	R	CAGCTCGTTATACACCCAGTCGGC	66.7	
Gapdh	F	ACCACAGTCCATGCCATCAC	62.7	450
	R	CACCACCCTGTTGCTGTAGCC	65.2	

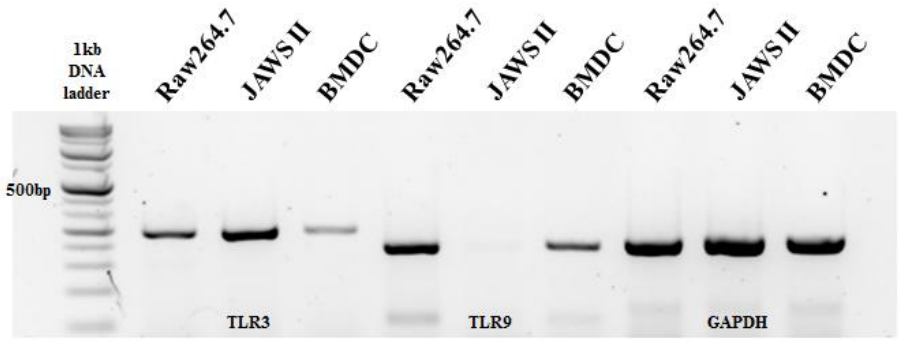

Figure 14. TLR expression

7) Cytokine ELISA

Raw264.7 and BMDC were cultured in 24-well plates prior to analysis. Then, cells were individually or simultaneously stimulated with CpG ODN (1.25, 2.5, 5 ug/ml) and poly I:C (12.5, 25, 50, 100 ug/ml) for 24 h. Culture supernatants were selectively collected by centrifugation at 1,500 rpm for 8 min. All murine cytokine levels were measured using mouse ELISA Complete Kit (IL-12 Cat. No. K0331139; TNF-α Cat. No. K0331186) (KOMA BIOTECH, Korea). Following manufacturer's instructions, ELISA was performed. After colorimetric detection was carried out at 450 nm, synergistic effect of TLR agonists was calculated following equation; Synergistic effect (%) = (Cytokine released by combinational stimulation)/(Single stimulation of CpG ODN + poly I:C)*100-100

3. Particle formation of M5BT with TLR agonists

1) Particle formulation via ionic interaction

M5BT was formulated with CpG ODN and poly I:C by ionic interaction. Solution suspended CpG ODN and poly I:C was dropped into solution containing M5BT under the mild vortexing for small volume (1 ml)or stirring for large volume (10 ml). Next, the solution was stirred for 5 to 10 min and shortly lyophilized for further use.

2) Morphology of particle

The physical properties of the particle were established using FE-SEM using SUPRA 55VP-SEM (Carl Zeiss, Germany) and TEM JEM1010 (JEOL, Japan). The lyophilized particle was

mounted on copper tape attached stub and coated subsequently with diffuse platinum. The particle size and surface conformation were confirmed by FE-SEM following various formulation (Table 12). TEM was used to determine the particle morphology in aqueous dispersion. The aqueous dispersion of the particle was dropped on a TEM copper grid and PTA negative staining ought to allow the strong contrast of samples against background was preceded by analysis. visualization was done using a Gatan Microscopy Suite Software, GMS 3 (Gatan Inc, USA).

Table 12. Particle formulation ratio

N/P ratio / Volume (ml)		0.1			1	
		M5BT (ug)	Adjuvant (ug)		M5BT (ug)	Adjuvant (ug)
10	A	660	1100	B	6600	1100
1	C	660	1100	D	6600	1100

4. *In vivo* immunization in mouse

1) Mice and immunization scheme

Age of 7 weeks of 5 female BALB/C mice purchased from the Samtako (Korea) were used per group in the study. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) and performed according to the regulation and guidelines for the care and use of laboratory animals (Seoul National University; ILAR 15-03-081).

Mice were intraperitoneally injected (i.p) with endotoxin removed M5BT and TLR agonists. The specific scheme and dose of materials were described in Table 13. Each group was injected by 17-gauge sterile hypodermic needle (Korea vaccine, Korea) with determined dose (Priming at 0 week; twice boosting at 1 and 2 week). Mouse serum obtained from the ophthalmic venous plexus of mice using collection tubes followed by subsequent centrifugation at 3,000 rpm for 2 min was carried out at 0 and 4 weeks of the experiment. At the last day, the spleen was isolated for further analysis.

Table 13. Immunization scheme

	Materials	Annotation	Dose of M5BT	Adjuvant
1	No Treatment	NT	-	-
2	iFMDV vaccine[1]	iFMDV	33 ug	Included
3	M5BT alone	M5BT	33 ug	-
4	M5BT and CFA	CFA	33 ug	CFA or IFA[2]
5	M5BT andCpG	CpG	33 ug	CpG 5 ug
6	M5BT and pIC	pIC	33 ug	pIC 50 ug
7	M5BT, CpG, pIC	Comb	33 ug	CpG 5 ug, pIC 50 ug

[1]Commercial iFMDV vaccine was used as positive control

[2]CFA was used at priming, IFA was used at boosting

2) Spleen isolation and cytokine ELISA

Spleen of immunized mice was isolated and prepared as single-cell suspension by gentle mechanical disruption using 40 um cell strainer (BD Falcon, USA). Next, the number of splenocytes was standardized preceded by the addition of ACK lysis buffer to remove red blood cells.

5 × 10^6 cells/well splenocytes from immunized mice were re-stimulated with M5BT (33 ug/ml) in 24-well plates containing RPMI 1640 supplemented with 10% FBS the and 1% P/S for 3 days. At the day 3, the culture supernatant was collected and measured Additionally, non-stimulated splenocytes of each group were cultured as a negative control.

The level of IFN-ɣ, IL-4 and IL-12 concentration of mice was analyzed by mouse ELISA Complete Kit (IFN-ɣ Cat. No. K0331138; IL-4 Cat. No. K0331144; IL-12 Cat. No. K0331139) (KOMA BIOTECH, Korea) as previously instructed.

3) Antibody response

(1) PI test

The functional antibody producing efficacy of adjuvants was assessed through PrioCHECK® FMDV type O (Prionics AG, Switzerland) following given protocol. Briefly, blood serum from immunized mice and given standard serum dissolved in provided ELISA buffer were reacted with pre-coated non-infectious FMDV type O antigen in test plates for 1 h at room temperature. After washing, conjugation was done for additional 1 h followed by sequential TMB and stop solution treatment.

Measured $O.D_{450}$ was converted as the following equation in order to calculate percentage inhibition (P.I)

$$P.I(\%) = 100 - \left[\frac{\text{corrected } O.D_{450} \text{ test sample}}{\text{corrected } O.D_{450} \text{ Max}} \right] \times 100$$

Samples displayed more than P.I 50% were interpreted having functional antibodies to FMD type O antigen.

(2) M5BT-specific ELISA

The M5BT-specific antibody titers were established through standard ELISA protocol. In brief, 96-well immunoplates were coated with M5BT at a concentration of 1 ug/ml in 0.05 M Carbonate-bicarbonate solution for 2h incubation at 37℃. Next, three times of washing with 1X PBS were performed followed by blocking (Blocking solution; 1% BSA in 1X PBS) for 1 h at room temperature. After the repeated washing steps, M5BT-coated plates was directly used for analysis.

Serum at 0 and 4 week from mice was loaded on each well through 5-fold serial dilution started from 1/100 diluted sample with blocking solution for 2 h of incubation at 37℃. After that plates were washed with PBST (PBST; 0.05% Tween 20) three times. Secondary HRP-conjugated anti-mouse IgG, IgG1 and IgG2a were diluted in 1% BSA added PBST (IgG and IgG1 were diluted at a 1:5000, IgG2a was diluted 1:2500) and the mixture was incubated for 1 h at room temperature. Plates were washed three times with PBST and TMB was added to develop blue

color at room temperature for 7 min and subsequently stopped by stop solution (Stop solution; 0.16 M H_2SO_4). The intensity was measured at a wavelength 450nm using an automatic ELISA plate reader TECAN Infinite 200 pro TECAN, Switzerland).

Carbonate-bicarbonate solution, BSA, Tween 20 and TMB (Sigma, USA), HRP-conjugated anti-mouse IgG and isotype of IgG (IgG1 and IgG2a) (Santa Cruz. USA) needed for this study was purchased from companies.

5. Statistical analysis

Quantified results were expressed as the mean ± standard deviation (SD). Statistical significance was assessed using t-test and a one-way analysis of variance (ANOVA) and post-hoc Tukey multiple comparison tests. All statistical analysis were carried out through GraphPad PRISM (GraphPad Software, USA) All statistical significance is denoted by $*p<0.05$, $**p<0.01$ and $***p<0.001$ between groups.

IV. Results and Discussion

1. Development of optimal medium for M5BT protein production in *E.coli*

1) Effect of medium composition on *E.coli* growth

Medium is the fundamental environment for *E.coli* producing recombinant protein. LB and TB broth are the conventional media used in laboratory shaken culture system to use recombinant protein from *E.coli*. As shown in Figure 15, it was revealed that these two media showed different *E.coli* growth curve over time. *E.coli* cultured in LB broth had low yield as it contained basic C and N sources ended below 6 $O.D_{600}$ at 12 h. On the contrary, TB broth helped *E.coli* to proliferate exhibiting high $O.D_{600}$ at the end. However, *E.coli* grown in nutritious TB broth showed extended lag phase compared to cultured *E.coli* in LB broth implied the needs for development of modified optimal medium for *E.coli* can overcome the weakness of conventional LB and TB broth.

Modified medium, T1B and T2B were mixed followed the compositions described in Table 8. Salts were supplemented in order to offer different buffer capacity and additional effect on microbial growth. Interestingly, *E.coli* cultured in modified medium showed enhanced $O.D_{600}$ than TB broth. In addition to, T2B broth had positive effect that shorten lag phase of *E.coli* contrasted with *E.coli* grown in T1B broth showed delayed growth performance (Figure 16) under the shaking flask conditions.

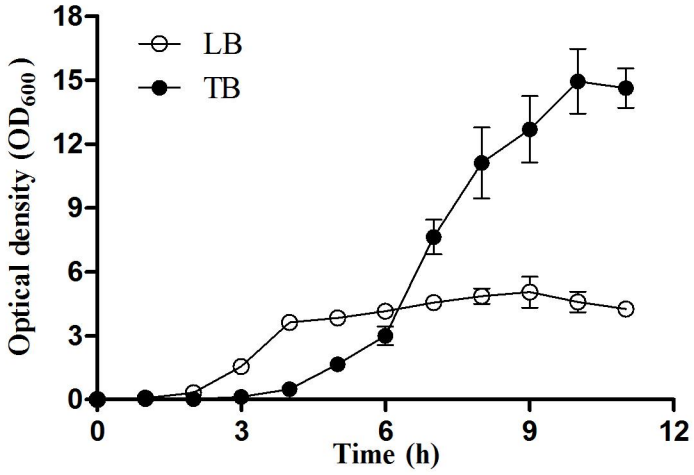

Figure 15. Growth curve of *E.coli* in LB and TB broth. $O.D_{600}$ of each medium over time without IPTG induction was indicated as open symbol (○) for LB broth; filled symbol (●) for TB broth.

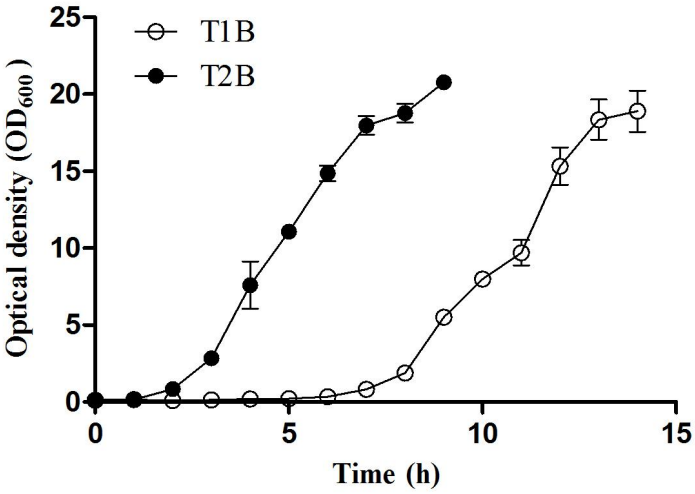

Figure 16. Growth curve of *E.coli* in T1B and T2B broth. OD_{600} of each medium over time without IPTG induction was indicated as open symbol (○) for T1B broth; filled symbol (●) for T2B broth.

2) Relative expression of soluble M5BT protein

The recombinant protein, M5BT produced from *E.coli* is artificial protein. Although the prokaryotic expression system enables the rapid and efficient production of recombinant protein, the absence of post-translational modification (PTM) may lead to the inclusion body formation. regarded as the main bottleneck of successful recombinant protein production. Hence, the expression of soluble M5BT from LB, TB and T2B broth was assessed through SDS-PAGE after IPTG induction in order to evaluate whether the modified medium helped *E.coli* to successfully make soluble M5BT compared to conventional medium. IPTG was used at 0.5 mM for *E.coli* grown in LB medium while 1 mM for *E.coli* cultured in TB and T2B broth at specific cell densities.

As a result, M5BT produced in LB broth had relatively high soluble expression among the medium. TB and T2B broth exhibited similar tendency with respect to producing less soluble M5BT than produced in LB broth (Figure 17). It was considered that *E.coli* cultured in a nutrient-enriched medium under the pressure for growth and protein production could result in the increased inclusion body accompanied with the increased soluble M5BT expression. Therefore, again, T2B broth was regarded as an ideal medium for recombinant protein production of *E.coli* which had high cell yield but not to be hampered in terms of soluble protein expression compared to TB broth (Figure 18).

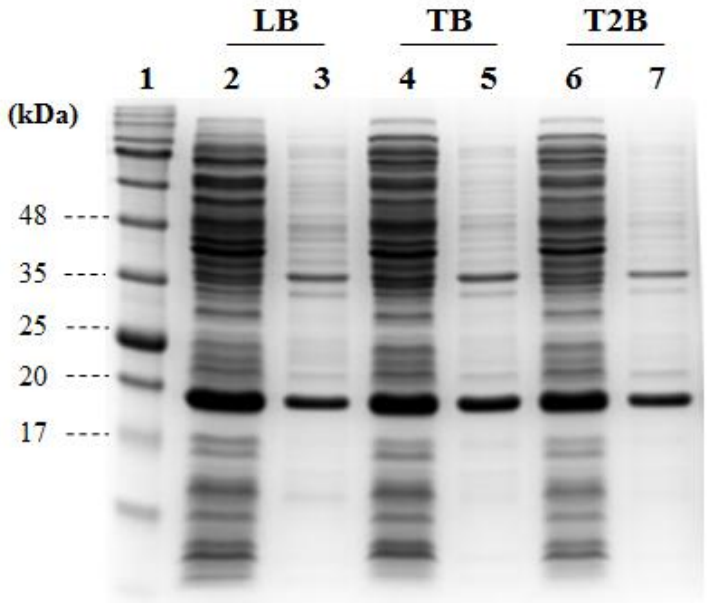

Figure 17. Relative expression of M5BT. Lanes: 1, protein ladder; 2, soluble fraction (LB); 3, insoluble fraction (LB); 4. soluble fraction (TB); 5, insoluble fraction (TB); 6, soluble fraction (T2B); 7, insoluble fraction (T2B). Molecular weight of M5BT, 19.1kDa; Sample used for TB and T2B was 4-fold diluted.

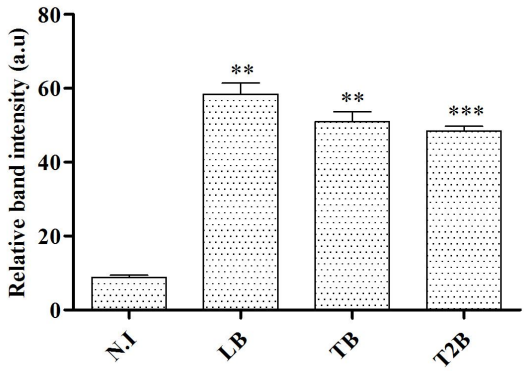

Figure 18. Quantification of relative M5BT expression. All .Data are presented as the means ± SD, n=3. *p<0.5, **p<0.01, ***p<0.001 between no induction and induced samples from different medium (LB, TB and T2B)

3) Quantification of purified M5BT

To prepare M5BT for FMDV multi-epitope vaccine, *E.coli* BL21 (DE3) was designed to express additional six consecutive histidine residues at the end of the sequences aimed to easy and efficient purification by Ni-NTA affinity chromatography (or His-tag affinity chromatography). Buffers were used for each step of chromatography as listed in Table 9. And fractions were collected to check the efficiency when Ni-NTA column used for purification. Especially, Eluted fraction was collected in the same volume (20 ml) to compare the total amount of purified M5BT produced from the different medium in a qualitative way using SDS-PAGE.

As expected, M5BT produced from TB and T2B broth showed high amounts of soluble M5BT over produced in LB broth. Moreover, it was revealed that modified T2B broth had much higher protein yield than TB broth had (Figure 19).

Then, the final concentration of M5BT was determined by the co-extinction efficient method preceded by dialysis and lyophilization of eluted M5BT solution. In accordance with the indirect concentration of M5BT visualized by SDS-PAGE results, T2B , the amount of M5BT from T2B broth had a concentration 3.74 times higher than LB broth and 1.29 times higher than TB broth, respectively (Table 14).

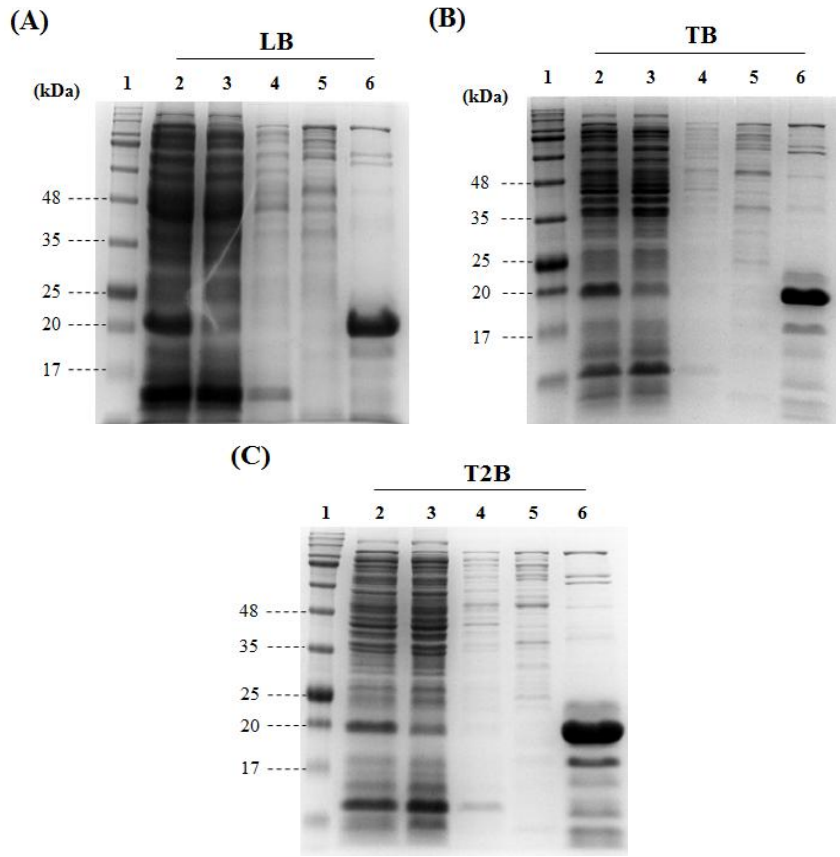

Figure 19. Purification of recombinant M5BT by Ni-NTA column. Each fraction from (A) LB , (B) TB and (C) T2B broth was presented. Lanes: 1, protein ladder; 2, SM, start material; 3, SFT, sample flow through; 4, W1, washed fraction 1; 5, W2, washed fraction 2; 6, EF, eluted fraction. 4 ml (LB broth) and 6 ml (TB and T2B broth) of resin volume was used. SM and EF of TB and T2B broth was 4-fold diluted; Molecular weight of M5BT, 19.1 kDa.

Table 14. Concentration of M5BT

Broth	LB	TB	T2B
Co-extinction efficient[1]	0.0946	0.271	0.35
Protein yield (mg/l)[2]	18	52	67.28

[1] $O.D_{280}$, X 1/10 dilution

[2] From co-extinction efficient calculation

Recombinant protein expression system in *E.coli* serves producers with a lot of advantages when it is designed for protein-based vaccine production (Tripathi et al., 2009). Application of efficient medium enables the host to produce bulk of soluble protein in short time. C, N sources are the basic and essential components of medium required in recombinant *E.coli* for the synthesis of protein needed to produce target protein as well as proliferate themselves. Salts added to medium have an impact to manipulate the subtle environment for *E.coli* growth. (Webb, 1968)

Here, it was found that T2B broth which was slightly modified from TB broth by addition of magnesium phosphate and change the ratio of potassium phosphate buffer system gave great benefit to *E.coli* in aspects of growth and production of target protein, It was confirmed that recombinant *E.coli* cultured in T2B broth grew faster and much more than others in the absence of IPTG induction. Under the IPTG induced conditions, cells grown in T2B culture system had a large population which was ready to produce target protein resulted in the high protein concentration as it determined by serial experiments.

Based on the above shaken flask culture results, this study implied compositions of culture medium can have effect on the microbial growth rate led to high cell and protein yield and suggested the insight that T2B broth can be applied in large-scale cultivation for recombinant protein manufacturing by achieving high cell densities

2. *In vitro* adjuvanticity validation of TLR agonists

1) *In vitro* stability and cell viability

Virus-like TLR agonist is a promising adjuvant partner for FMDV multi-epitope vaccine by stimulating innate immune system as these molecules mimic the viral pathogen. Especially, the classic virus-like TLR agonists, CpG ODN and poly I:C recognized by endosomal TLRs have been shown to stimulate immune response synergistically. It has been elucidated that amplified signal from different signal molecule pathway contributed the synergistic effects between CpG ODN and poly I:C. However, the effect of simultaneous stimulation of CpG ODN and poly I:C as adjuvants when it is combined with the multi-epitope vaccine is still unclear.

To elucidate the adjuvanticity of CpG ODN and poly I:C and its synergistic effect, *in vitro* validations based on murine immune cell line were performed. *in vitro* stability and cell viability were done prior to cytokine production to determine optimal dose and treatment time for *in vitro* analysis.

First, CpG ODN and poly I:C were individually incubated in the stock solution and medium and partial fractions at 0, 1, 6 and 24 h were collected for analysis. On the basis of band intensity, it was confirmed that both TLR agonists are preserved from degradation until 24 h (Figure 20).

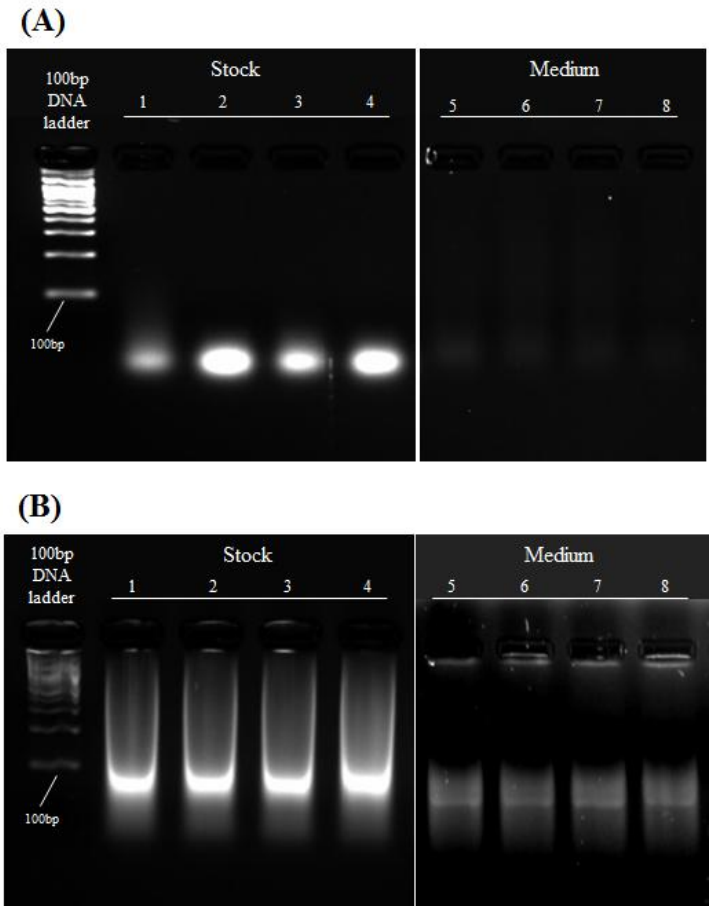

Figure 20. *In vitro* stability of TLR agonists in stock and medium. Samples were incubated in endotoxin free water (CpG ODN) or DEPC treated water (poly I:C), respectively (250 ng/ul) and DMEM (50 ng/ul) at 37℃ up to 24 h. Stability of (A) CpG ODN; (B) poly I:C collected at each time point (0, 1, 6 and 24 h) were presented as relative band intensity in 3% agarose gel.

Next, cytotoxicity stimulated with CpG ODN and poly I:C on Raw 264.7 cells was assessed by MTT assay. It has been known that excessive inflammatory cytokine stimuli induced by virus-like TLR agonists on cells may lead the severe inflammation such as sickness and fever in *in vivo* murine model . Therefore, the ranges of CpG ODN and poly I:C had to be determined for the purpose of optimal dose decision.

As shown in Figure 21, CpG ODN did not have negative effect on Raw 264.7 cells at high concentration (20 ug/ml) compared to untreated group (Figure 21. A) but murine macrophage stimulated with poly I:C exhibited decreased cell viability from 50 ug/ml (Figure 21. B). Surprisingly, the cytotoxic effect induced from poly I:C was alleviated when CpG ODN and poly I:C were treated together and furthermore, this combinational stimulation of TLR agonists enhanced Raw 264.7 cell proliferation at low concentration (CpG ODN 2.5 ug/ml and poly I:C 25 ug/ml) (Figure 21. C).

Taken together, these results implied the simultaneous CpG ODN and poly I:C stimulation had positive and powerful adjuvanticity on innate immunity by modulating cytotoxicity derived from poly I:C and activating cell proliferation.

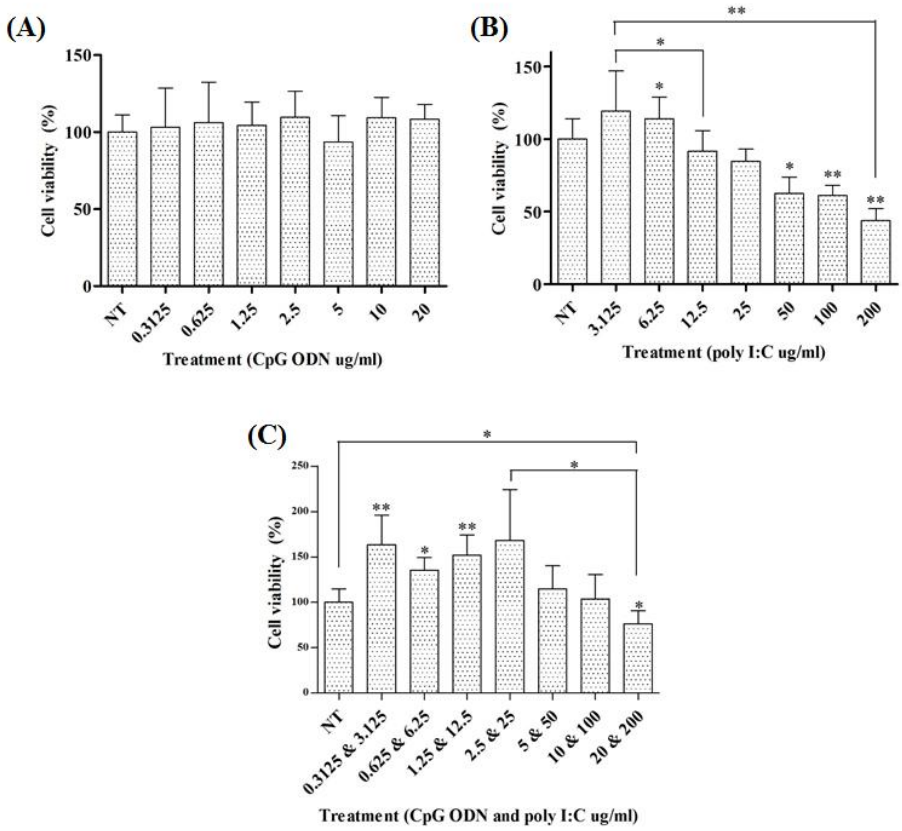

Figure 21. Cell viability after treating TLR agonists on Raw 264.7 cell line. Raw264.7 cells stimulated with (A) CpG ODN, (B) poly I:C, (C) Combination (CpG ODN and poly I:C) at various concentrations ranging from 0.3125 to 20 ug/ml (CpG ODN) and 3.125 to 200 ug/ml (poly I:C). for 24 h. All data are presented as the means ± SD, n=5. (*$p<0.05$, **$p<0.01$, ***$p<0.001$ one-way ANOVA)

2) mRNA expression of pro- and anti-inflammatory cytokine in Raw264.7 cell

Detection of pro-and anti-inflammatory cytokine mRNA from Raw264.7 cells was estimated with the intention of the investigating the modulation effect of CpG ODN and poly I:C in terms of synergistic cytokine production. Expression of TLR3 and TLR9 on various cell line were confirmed by reverse transcription technique (Figure 14) and cytokine-specific primers were designed prior to the study (Table 11).

As shown in Figure 22, pro-inflammatory cytokines; IL-1β, IL-6 and TNF-α increased in the group treated with CpG ODN and poly I:C together. Particularly, IL-6 had significantly improved mRNA expression compared to individually treated groups (Figure 22. B) and the expression level of typical anti-inflammatory cytokine, IL-10 was also increased in a dose-dependent manner (Figure 22. D)

In an aspect of immunology, cytokines have an important role in not only innate immunity but also adaptive immunity as it provokes a non-specific response against infection and facilitates specific response to antigen. Especially, cytokines secreted from macrophage were known to have abilities to recruit other immune cells such as macrophages and NK cells involving first line defense mediator in innate immune response and dendritic cells offering adaptive immune response via T cell interactions.

Increased IL-1β and TNF-α expression levels implied that the combinational stimulation of TLR agonists may contribute to creating the alert environment against possible pathogen infection. Also, one of the roles of multi-functional cytokine IL-6 has been

known to contribute to cytotoxic T lymphocyte (CTL) differentiation. (Ming et al., 1992). The dose-dependent expression of IL-10 from Raw264.7 cells was interpreted as the result of multi-layered and complex interaction between cytokine signals. Taken together, these results gave us an insight how CpG ODN and poly I:C can efficiently modulate the innate immunity as ideal adjuvant partners of FMDV multi-epitope vaccine.

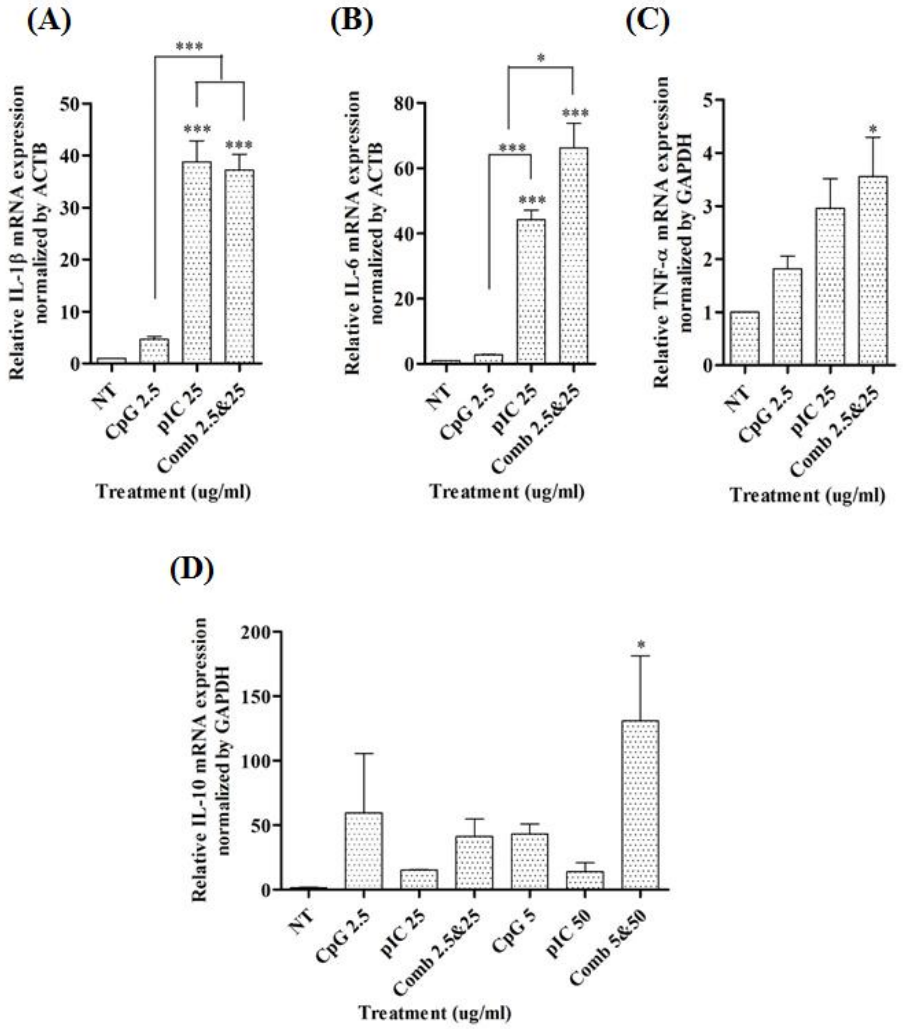

Figure 22. Effect of CpG ODN and poly I:C on pro- and anti-inflammatory cytokine expression. Raw264.7 cells were treated with CpG ODN and poly I:C (Comb; CpG ODN and poly I:C) and total RNA was extracted for the quantification of cytokine mRNA levels, including (A) IL-1β; (B) IL-6; (C) TNF-α (D) IL-10. All data are presented as the means ± SD, n=3. (*p<0.05, **p<0.01, ***p<0.001 one-way ANOVA)

3) Th1 response related cytokine ELISA

Based on the synergistically increased cytokine expression in Raw264.7, cytokine ELISA was performed to validate whether simultaneous stimulus of CpG ODN and poly I:C induce Th1 type immune response for the defense against viral infection. Murine macrophage cell line (Raw 264.7) and dendritic cells differentiated from bone marrow (BMDC) were used for the analysis. Prior to use, BMDC was evaluated through phenotype analysis by flow cytometry and RT-PCR for IL-12 ELISA (Figure 13 and 14).

Accordance with the previous results, as shown in Figure 23, the synergistic level of TNF-α from Raw264.7 cells was confirmed from at a low concentration (CpG ODN 1.25 ug/ml and poly I:C 12.5 ug/ml). Further, It was revealed that combinational stimulation treated to BMDC showed significantly increased IL-12 production in a synergistic way (Figure 24) even though secreted IL-12 was not found in Raw264.7 (Data not shown).

In addition, the synergistic effect of TLR agonists boosting Th1 response was figured out by measuring secreted IFN-γ from primary spleen cell culture supernatant. As expected, IFN-γ was significantly increased in the combination group (Figure 25).

IL-12 and IFN-γ are the pivotal cytokines providing guided signal from DCs to naive helper T cell towards Th1 polarized way. Th1 cells are thought to induce a specific immune response against intracellular pathogens like a virus and also attack the cancer cells (Kidd, 2003).

Taken above results together, a combination of CpG ODN and poly I:C showed various advantages of immune cell proliferation with mitigated cytotoxicity and increased pro-and

anti-inflammatory cytokine release having great potential as suitable immunostimulatory adjuvant aims to activate innate immune response and modulate lineage of T cell differentiation. Thus, the strategy that CpG ODN and poly I:C are combined with FMDV multi-epitope vaccine, M5BT can be the way to overcome weak immunogenicity derived from its partial virulent structure and furthermore induce desirable immune response against viral infection.

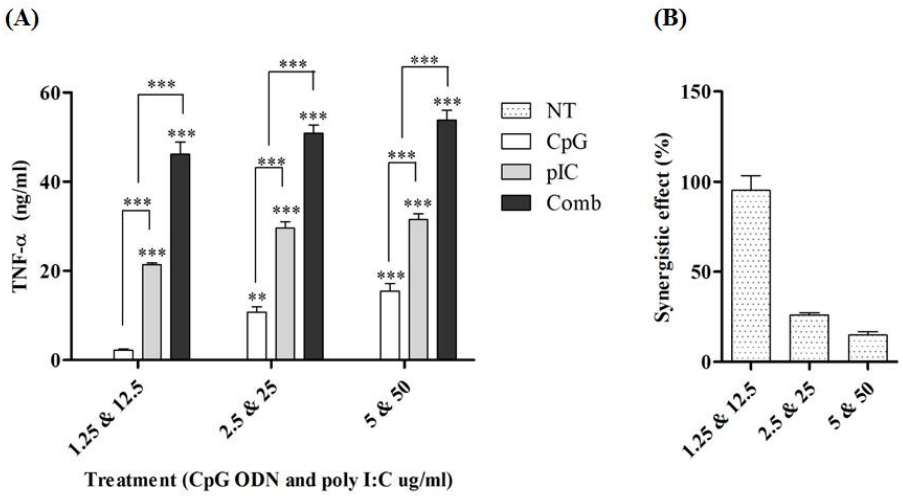

Figure 23. TNF-α levels in Raw264.7 cells. Cells were treated with CpG ODN and poly I:C at various concentrations ranging from 1.25 to 5 ug/ml (CpG ODN) and 12.5 to 50 ug/ml (poly I:C). (A) Secreted TNF-α level (ng/ml); (B) Calculated synergistic effect; Synergistic effect (%) = (Cytokine released by combinational stimulation)/(Single stimulation of CpG + pIC)*100-100. All data are presented as the means ± SD, n=3. (*p<0.05, **p<0.01, ***p<0.001 one-way ANOVA)

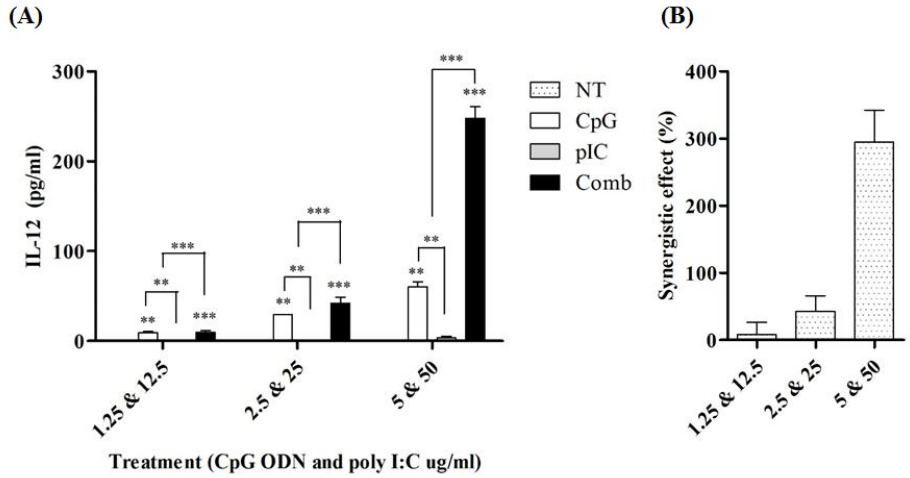

Figure 24. IL-12 levels in BMDCs. Cells were treated with CpG ODN and poly I:C at various concentrations ranging from 1.25 to 5 ug/ml (CpG ODN) and 12.5 to 50 ug/ml (poly I:C). (A) Secreted IL-12 level (pg/ml); (B) Calculated synergistic effect; Synergistic effect (%) = (Cytokine released by combinational stimulation)/(Single stimulation of CpG + pIC)*100-100. All data are presented as the means ± SD, n=3. (*p<0.05, **p<0.01, ***p<0.001 one-way ANOVA)

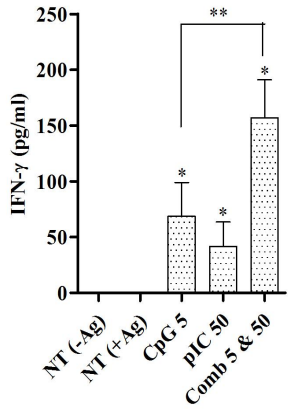

Figure 25. IFN-γ levels in splenocytes. Secreted IFN-γ level (pg/ml) from Cells stimulated with CpG ODN (5 ug/ml), poly I:C (50 ug/ml) and combination of CpG ODN and poly I:C (Comb); Calculated synergistic effect was 42.2 %; All data are presented as the means ± SD, n=3. (*p<0.05, **p<0.01, ***p<0.001 one-way ANOVA)

3. Particle formation of M5BT with TLR agonists

To make particulate M5BT, purified M5BT protein preceded by endotoxin removal was formulated with CpG ODN and poly I:C via ionic interaction. Positively charged M5BT protein is relatively positive charged at neutral pH because of the positive amino acid residues (Arg, Lys) described in Table 7. On the contrary, TLR agonists comprised of nucleic acid present negative charges from phosphate backbone. By applying this interaction, the particulate M5BT with two TLR agonists was simply formed.

Interestingly, it was found that the successful formation of the particulate M5BT was sensitively depended on the reaction volumes and ratio between the amine group of M5BT protein (N) and the phosphate group of TLR agonists (P) (N/P ratio) revealed at Figure 26. And among the candidates, N/P ratio of 0.1 reacted in 1 ml of 1X PBS was determined for desirable formula to produce the particulate M5BT confirmed by SEM and TEM (Figure 27)

The degradation of protein-based vaccine at *in vivo* immunization was the major hurdle ought to conquer for efficient vaccine delivery. and here, this simple strategy using ionic interaction force gave numerous benefits. First, the material loss during encapsulation procedure using polymeric carrier will not be occur and therefore, each component does not necessarily need to be quantified.

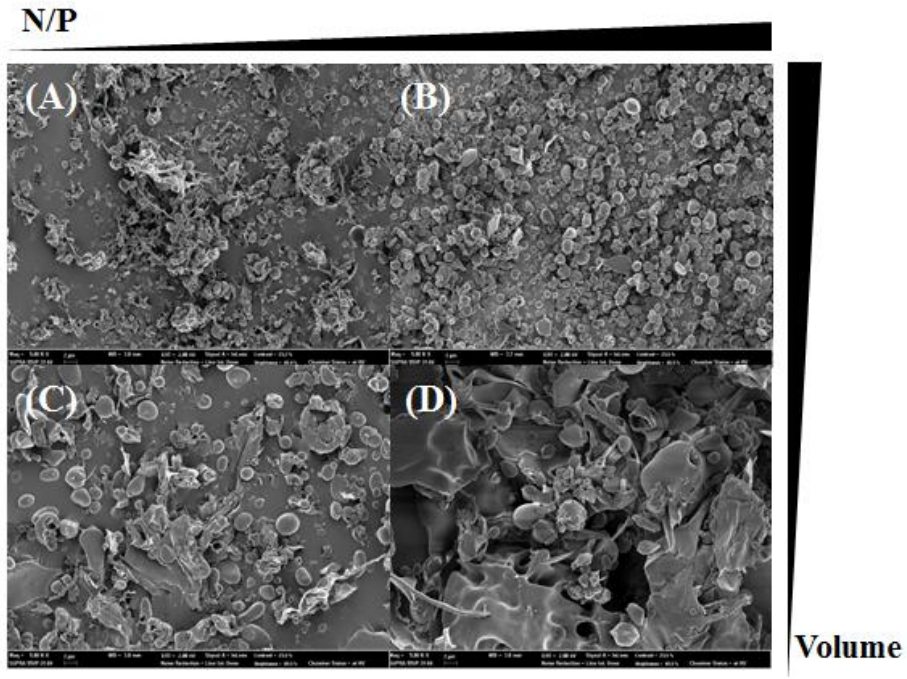

Figure 26. Particle morphology depends on reaction volume and N/P ratio. The formulations were done following Table X. Panels. (A) N/P ratio = 0.1, reaction volume 10 ml; (B) N/P ratio = 1, reaction volume 10 ml; (C) N/P ratio = 0.1, reaction volume 1 ml; (D) N/P ratio = 1, reaction volume 1 ml was analyzed by FE-SEM. (Magnification: 5,000X).

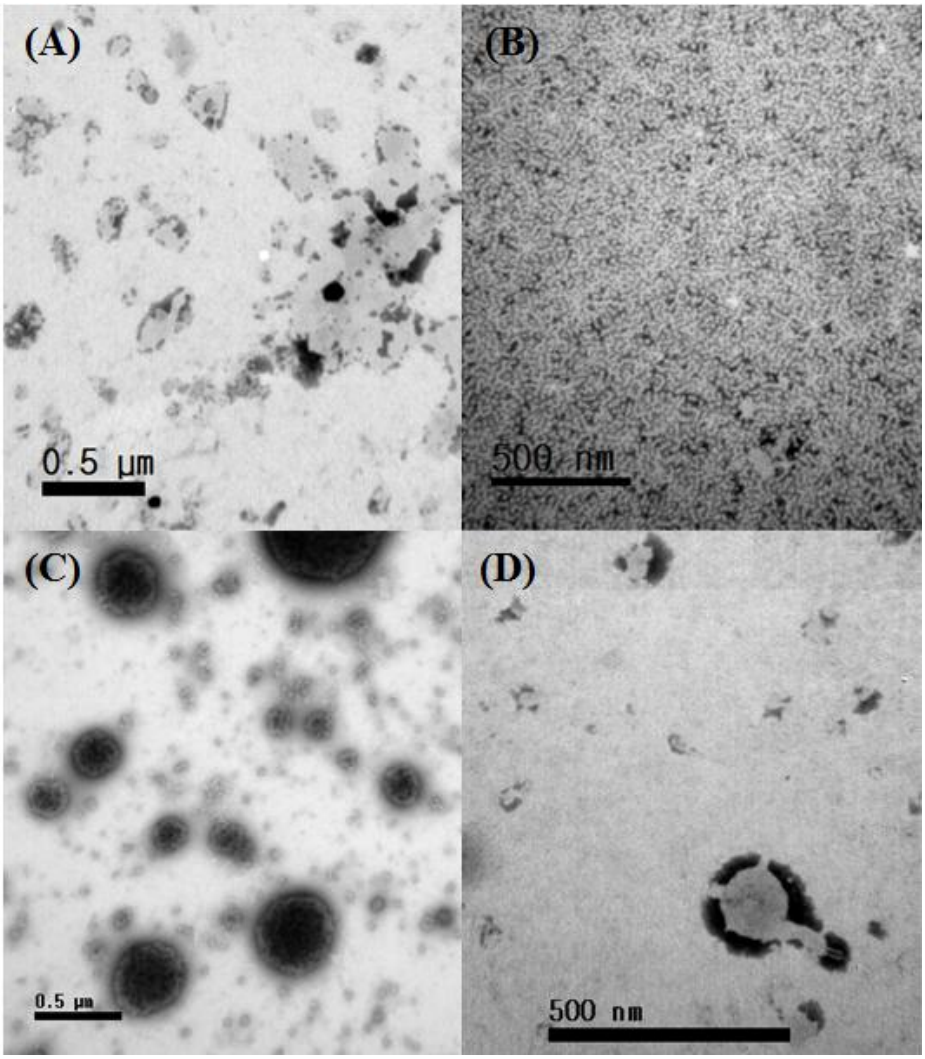

Figure 27. TEM images of particulate M5BT. (A) N/P ratio of 0.3; (B) N/P M5BT alone; (C) N/P ratio of 0.1; (D) N/P ratio of 1. (bar scale = 0.5 um).

4. *In vivo* mouse immunization

1) M5BT specific ELISA

To evaluate the adjuvanticity of CpG ODN and poly I:C, mice were immunized with iFMDV, M5BT alone, M5BT with CFA, CpG ODN alone, poly I:C alone and CpG ODN and poly I:C via intraperitoneal route (i.p) for 4 weeks.

Competitive ELISA against FMDV type O was conducted using antisera at 4 week. And M5BT-specific antibody titers were analyzed using immunized mouse serum at 0 and 4 week. and next, the IgG isotype, IgG_1 and IgG_{2a} were also analyzed to determine whether the different adjuvant combinations with multi-epitope vaccine M5BT led to specific types of adaptive immune response represented in IgG_1 for Th2 response and IgG_{2a} for Th1 response.

As results indicated, any groups did not pass the threshold (PI of 50 %) except iFMDV immunized group. but the group used poly I:C for adjuvant showed the partial protection compared to rest of the groups (Figure 28. A). In terms of M5BT-specific antibody response, groups adjuvanted with poly I:C only and CpG and poly I:C together showed elevated M5BT-specific IgG titers to the extent of the positive control groups, iFMDV and M5BT with CFA (Figure 28. B). The titers of M5BT-specific IgG_1 and IgG_{2a} were also showed similar tendency as we observed in M5BT-specific IgG results (Figure 29).

These results indicated that poly I:C and the combination of CpG ODN and poly I:C had potential adjuvanticity in terms of generating humoral immune response .against FMDV by providing comparable amounts of total IgG and IgG isotype

compared to commercial inactivated vaccine (iFMDV) and powerful laboratory adjuvant (M5BT with CFA).

It has been studied that the effect of subunit vaccine in mouse model can be tailored by different combination of adjuvants and delivery routes and this fact was confirmed in comparison with the previous immunization results using intramuscular route. The elevated M5BT-specific IgG response by change of injection methods from intraperitoneal route (i.p) to intramuscular injection (i.m) was found (Data not shown)

Moreover, nevertheless the dose was half-reduced from the previous immunization, the enhanced humoral immune response was achieved by particulate M5BT with CpG ODN and poly I:C. An overdose on TLR agonists might induce excessive inflammatory signal resulted in sickness or fever in mouse experiments. Thus, these results implied this combinational adjuvant system efficiently induced proper antibody response but cutting down the potential hazard of inflammation.

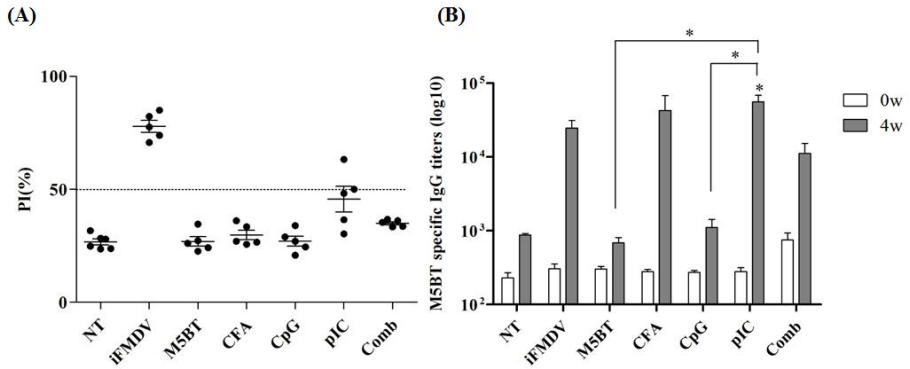

Figure 28. ELISA for *in vitro* detection of antibodies. serum from immunized mice was analyzed using (A) FMDV serotype O antigen and (B) pre-coated M5BT ELISA. All data are presented as the means ± SD, n=5. (*p<0.05, **p<0.01, ***p<0.001 one-way ANOVA)

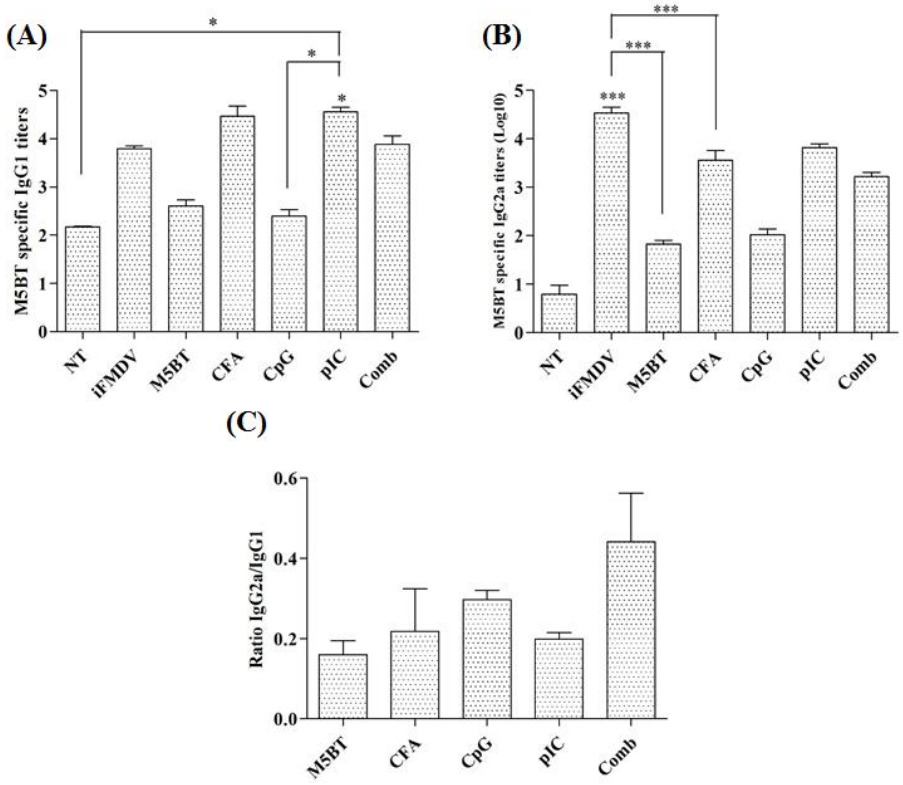

Figure 29. M5BT-specific IgG isotype titers. Anti-M5BT serum IgG levels at 0 and 4 weeks were measured using M5BT-coated ELISA. (A) M5BT-specific IgG_1; (B) M5BT-specific IgG_{2a}; (C) Ratio of IgG_{2a} and IgG_1. All data are presented as the means ± SD, n=5. (*p<0.05, **p<0.01, ***p<0.001 one-way ANOVA)

2) Cytokine ELISA

To figure out the memory response according to types of helper T cell immunity, cytokine ELISA detecting IL-4 and IFN-ɣ was conducted using splenocytes culture supernatant with or without antigen re-stimulation for 3 days.

The splenocytes immunized with iFMDV produced high levels of IL-4 than any other groups and poly I:C adjuvanted group followed. This result exhibited the similar tendency with the antigen-specific antibody response results described above (Figure 28. A). Interestingly, the levels of IFN-ɣ was significantly increased in CpG ODN alone and both CpG ODN and poly I:C adjuvanted group (Figure 28. B) contrary to previous antibody results but had a coincidence with the *in vitro* IFN-ɣ ELISA result. The detected IL-12 also showed increment but not significant in adjuvanted groups (Figure 30).

These results gave us a insight that CpG ODN and poly I:C adjuvant combination induced T cell-mediated adaptive immune response in a Th1-polarized way although the tendency was not correlated with the antibody titer results. The possible reason of unmatched tendency between IgG titers and cytokine levels was the different guiding adjuvant effect depends on types of CpG ODN. CpG ODN sequences used in this study was classified as A class which is known for inducing IFN-α secretion from plasmacytoid DC and less activating B cell response than B Class CpG ODN. So, a low dose of CpG ODN (5 ug per mouse) could not give sufficient signals to B cell required for optimal antibody production as it shown in Figure 25. However, A class CpG ODN exhibited high adjuvanticity with respect to IFN-ɣ production (Figure 30. B).

The synergistic effects of CpG ODN and poly I:C were evaluated through *in vitro* and *in vivo* analysis. and all things considered, the adjuvanticity of two TLR agonists were speculated to enhance proliferation of sentinel immune cell like macrophage and induce a broad spectrum of pro- and anti-inflammatory cytokine production followed by polarized activation of type 1 helper T cell interacted with dendritic cells.

Indeed, it seemed that low immunogenicity of protein-based vaccine can be overcome when it combined with CpG ODN and poly I:C together by providing properly oriented protective immunity against potential FMDV infection. Furthermore, The key anti-viral cytokine like IFN-γ can contribute the activation of CTL for defense.

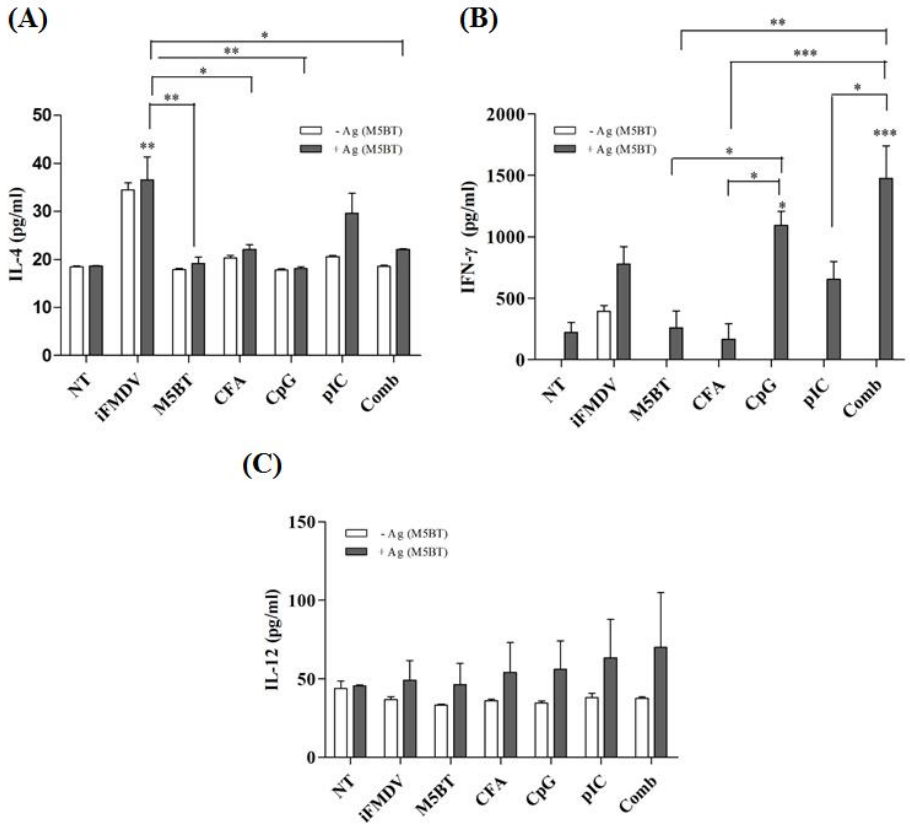

Figure 30. Cytokine production from antigen re-stiumulated murine splenocytes. Splenocytes ($1x10^7$ cells/ml) from immunized mice were incubated with 33 ug of M5BT for 3 days. (A) IL-4; (B) IFN-ɣ; (C) IL-12. All data are presented as the means ± SD, n=5. (*p<0.05, **p<0.01, ***p<0.001 one-way ANOVA)

V. Conclusion and Further prospects

Vaccine production in *E.coli* expression system enables to take rapid action for FMD recurrences. However, though this strategy offers benefits to research and livestock industry, there are two major hurdles to overcome. First, sufficient amount of the soluble target vaccine protein should be prepared for commercial use. and Next, efficient adjuvant combinations need to be suggested to improve weak immunogenicity of the protein-based vaccine.

In this regards, this study has significance in the development of optimal medium composition for *E.coli* growth and validation of CpG ODN and poly I:C combined adjuvant exerted immune response.

As results explained, it was found that modified terrific broth (T2B) promoted a desirable environment for *E.coli* in early stages of bacterial growth resulted in reduced lag phase and further, The total amount of cell grown in T2B culture was increased than others.

Because of the absence of post-translational modification in *E.coli*, designed protein is susceptible to be expressed in inclusion body, the aggregates of unfolded protein. To consider this, T2B was suggested as proper medium for producing recombinant vaccine in *E.coli* culture.

Next, i*n vitro* and *in vivo* adjuvanticity validation for CpG ODN and poly I:C indicated TLR synergy was found in innate immune response. In particular, cell proliferation and activation (e.g. elevated IL-1β, IL-6, TNF-α and IL-12 levels).

In addition to this, antigen-specific Th1-polarized immune response was found from mouse immunization results. The combinational stimulation of CpG ODN and poly I:C offered great benefits to host by inducing significantly high levels of IFN-γ release. This protein is represented as a hallmark of Th1 type immune response and anti-viral effect. And moreover, the simultaneous stimulation of adjuvant could exert antigen-specific antibody in certain extents. These results what was not induced by individual adjuvant stimulation. Therefore, CpG ODN and poly I:C was suggested for promising adjuvant combination with FMD vaccine based on results.

VI. Literature Cited

Aiyer Harini, P., Ashok Kumar, H. G., Praveen Kumar, G., & Shivakumar, N. (2013). An Overview of Immunologic Adjuvants-A Review. Journal of Vaccines & Vaccination.

Amadori, M., Volpe, G., Defilippi, P., & Berneri, C. (1997). Phenotypic features of BHK-21 cells used for production of foot-and-mouth disease vaccine. Biologicals, 25(1), 65-73.

Awate, S., Babiuk, L. A. B., & Mutwiri, G. (2013)a. Mechanisms of action of adjuvants. Frontiers in immunology, 4, 114.

Berryman, S., Clark, S., Monaghan, P., & Jackson, T. (2005). Early events in integrin αvβ6-mediated cell entry of foot-and-mouth disease virus. Journal of virology, 79(13), 8519-8534.

Bhardwaj, N., Gnjatic, S., & Sawhney, N. B. (2010). TLR AGONISTS: Are They Good Adjuvants?. Cancer journal (Sudbury, Mass.), 16(4), 382.

Bijker, M. S., Melief, C. J., Offringa, R., & Van Der Burg, S. H. (2007). Design and development of synthetic peptide vaccines: past, present and future. Expert review of vaccines, 6(4), 591-603.

Blanco, E., Cubillos, C., Moreno, N., Bárcena, J., de la Torre, B. G., Andreu, D., & Sobrino, F. (2013). B epitope multiplicity and

B/T epitope orientation influence immunogenicity of foot-and-mouth disease peptide vaccines. Clinical and Developmental Immunology, 2013.

Botos, I., Segal, D. M., & Davies, D. R. (2011). The structural biology of Toll-like receptors. Structure, 19(4), 447-459.

Brito, B. P., Rodriguez, L. L., Hammond, J. M., Pinto, J., & Perez, A. M. (2015). Review of the Global Distribution of Foot and Mouth Disease Virus from 2007 to 2014. Transboundary and emerging diseases.

Brown, F., Hyslop, N. S. G., Crick, J., & Morrow, A. W. (1963). The use of acetylethyleneimine in the production of inactivated foot-and-mouth disease vaccines. Journal of Hygiene, 61(03), 337-344.

Brun, A., Bárcena, J., Blanco, E., Borrego, B., Dory, D., Escribano, J. M., Gall-Reculé, G. L., & Dixon, L. K. (2011). Current strategies for subunit and genetic viral veterinary vaccine development. Virus Research, 157(1), 1-12.

Burman, A., Clark, S., Abrescia, N. G., Fry, E. E., Stuart, D. I., & Jackson, T. (2006). Specificity of the VP1 GH loop of foot-and-mouth disease virus for αv integrins. Journal of virology, 80(19), 9798-9810.

Casewell, M., Friis, C., Marco, E., McMullin, P., & Phillips, I.

(2003). The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. Journal of antimicrobial chemotherapy, 52(2), 159-161.

Chuai, X., Chen, H., Wang, W., Deng, Y., Wen, B., Ruan, L., & Tan, W. (2013). Poly (I:C)/alum mixed adjuvant priming enhances HBV subunit vaccine-induced immunity in mice when combined with recombinant adenoviral-based HBV vaccine boosting. PloS one, 8(1), e54126.

Cottam, E. M., Wadsworth, J., Shaw, A. E., Rowlands, R. J., Goatley, L., Maan, S., Maan, N. S., Mertens, P. P., Ebert, K., Li, Y., Ryan, E. D., Juleff, N., Ferris, N. P., Wilesmith, J. W., Haydon, D. T., King, D. P., Paton, D. J., & Knowles, N. J. (2008). Transmission pathways of foot-and-mouth disease virus in the United Kingdom in 2007. PLoS Pathog, 4(4), e1000050.

Crowe, J., Dobeli, H., Gentz, R., Hochuli, E., Stiiber, D., & Henco, K. (1994). 6xffis-Ni-NTA Chromatography as a Superior Technique in Recombinant Protein Expression/Purification. Protocols for Gene Analysis, 371-387.

Fioretti, D., Iurescia, S., Fazio, V. M., & Rinaldi, M. (2010). DNA vaccines: developing new strategies against cancer. BioMed Research International, 2010.

Fry, E. E., Lea, S. M., Jackson, T., Newman, J. W., Ellard, F. M., Blakemore, W. E., Abu-Ghazaleh, R., Samuel, A., King, A. M., &

Stuart, D. I. (1999). The structure and function of a foot and mouth disease virus - oligosaccharide receptor complex. The EMBO Journal, 18(3), 543-554.

Gil, F., Brun, A., Wigdorovitz, A., Catalá, R., Martí nez-Torrecuadrada, J. L., Casal, I., Salinas, J., Borca, M. V., & Escribano, J. M. (2001). High yield expression of a viral peptide vaccine in transgenic plants. FEBS letters, 488(1-2), 13-17.

Grubman, M. J., & Baxt, B. (2004). Foot-and-mouth disease. Clinical microbiology reviews, 17(2), 465-493.

Hacein-Bey-Abina, .S., Von Kalle, C., Schmidt, M., McCormack, M. P., Wulffraat, N., Leboulch11, P., Lim, A., Osborne, C. S., Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., Fraser, P., Cohen, J. I., de Saint Basile, G., Alexander, I., Wintergerst, U., Frebourg, T., Aurias, A., Stoppa-Lyonnet. D., Romana, S., Radford-Weiss, I., Gross, F., Valensi, F., Delabesse, E., Macintyre, E., Sigaux, F., Soulier, J., Leiva, L. E., Wissler, M., Prinz, C., Rabbitts, T. H., Le Deist, F., Fischer, A., & Cavazzana-Calvo, M.. (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. science, 302(5644), 415-419.

Hafner, A. M., Corthésy, B., & Merkle, H. P. (2013). Particulate formulations for the delivery of poly (I: C) as vaccine adjuvant. Advanced drug delivery reviews, 65(10), 1386-1399.

Heegaard, P. M., Boas, U., & Sorensen, N. S. (2009). Dendrimers for vaccine and immunostimulatory uses. A review. Bioconjugate Chemistry, 21(3), 405-418.

Hem, S. L., & HogenEsch, H. (2007). Relationship between physical and chemical properties of aluminum-containing adjuvants and immunopotentiation. Expert review of vaccines, 6(5), 685-698.

Ishii, K. J., Takeshita, F., Gursel, I., Gursel, M., Conover, J., Nussenzweig, A., & Klinman, D. M. (2002). Potential Role of Phosphatidylinositol 3 Kinase, rather than DNA-dependent Protein Kinase, in CpG DNA - induced Immune Activation. The Journal of experimental medicine, 196(2), 269-274.

Ishii, K. J., & Akira, S. (2006). Innate immune recognition of, and regulation by, DNA. Trends in immunology, 27(11), 525-532.

Jamal, S. M., & Belsham, G. J. (2013). Foot-and-mouth disease: past, present and future. Veterinary research, 44(1), 1.

Jung, S. N., Kang, S. K., Yeo, G. H., Li, H. Y., Jiang, T., Nah, J. W., Bok, J. D.,. & Choi, Y. J. (2015). Targeted delivery of vaccine to dendritic cells by chitosan nanoparticles conjugated with a targeting peptide ligand selected by phage display technique. Macromolecular bioscience, 15(3), 395-404.

Kersten, G. F., & Crommelin, D. J. (2003). Liposomes and iscoms.

Vaccine, 21(9), 915-920.

Kidd, P. (2003). Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Alternative Medicine Review, 8(3), 223-246.

Klinman, D. M. (2004). Immunotherapeutic uses of CpG oligodeoxynucleotides. Nature Reviews Immunology, 4(4), 249-259.

Krummen, M., Balkow, S., Shen, L., Heinz, S., Loquai, C., Probst, H. C., & Grabbe, S. (2010). Release of IL-12 by dendritic cells activated by TLR ligation is dependent on MyD88 signaling, whereas TRIF signaling is indispensable for TLR synergy. Journal of leukocyte biology, 88(1), 189-199.

Kumru, O. S., Joshi, S. B., Smith, D. E., Middaugh, C. R., Prusik, T., & Volkin, D. B. (2014). Vaccine instability in the cold chain: mechanisms, analysis and formulation strategies. Biologicals, 42(5), 237-259.

Lan, J., Deng, Y., Chen, H., Lu, G., Wang, W., Guo, X., Lu, Z., Gao, G. F.,. & Tan, W. (2014). Tailoring subunit vaccine immunity with adjuvant combinations and delivery routes using the Middle East respiratory coronavirus (MERS-CoV) receptor-binding domain as an antigen. PloS one, 9(11), e112602.

Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K. A., Monks, B. G., Knetter, C. F., Lien, E., Nilsen, N. J., Espevik, T., &

Golenbock, D. T. (2004). TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nature immunology, 5(2), 190-198.

Lee, J. W., Hwang, J. H., & Yoe, H. (2011, December). Design of integrated control system for preventing the spread of livestock diseases. In International Conference on Future Generation Information Technology (pp. 169-173). Springer Berlin Heidelberg.

Lee, S., & Nguyen, M. T. (2015). Recent advances of vaccine adjuvants for infectious diseases. Immune network, 15(2), 51-57.

Li, W., Joshi, M. D., Singhania, S., Ramsey, K. H., & Murthy, A. K. (2014). Peptide vaccine: Progress and challenges. Vaccines, 2(3), 515-536.

Loeffler, F., & Frosch, P. (1897). Summarischer Bericht über die Ergebnisse der Untersuchungen der Kommission zur Erforschung der Maul-und Klauenseuche bei dem Institut für Infektionskrankheiten in Berlin. Dt Med Wschr, 98, 80-84.

Longjam, N., Deb, R., Sarmah, A. K., Tayo, T., Awachat, V. B., & Saxena, V. K. (2011). A brief review on diagnosis of foot-and-mouth disease of livestock: conventional to molecular tools. Veterinary medicine international, 2011.

Mannhalter, J. W., Neychev, H. O., Zlabinger, G. J., Ahmad, R., & Eibl, M. M. (1985). Modulation of the human immune response

by the non-toxic and non-pyrogenic adjuvant aluminium hydroxide: effect on antigen uptake and antigen presentation. Clinical and experimental immunology, 61(1), 143.

Marrack, P., McKee, A. S., & Munks, M. W. (2009). Towards an understanding of the adjuvant action of aluminium. Nature Reviews Immunology, 9(4), 287-293.

Martins, K. A., Bavari, S., & Salazar, A. M. (2015). Vaccine adjuvant uses of poly-IC and derivatives. Expert review of vaccines, 14(3), 447-459.

Meeusen, E. N., Walker, J., Peters, A., Pastoret, P. P., & Jungersen, G. (2007). Current status of veterinary vaccines. Clinical microbiology reviews, 20(3), 489-510.

Meylan, E., Tschopp, J., & Karin, M. (2006). Intracellular pattern recognition receptors in the host response. Nature, 442(7098), 39-44.

Ming, J. E., Steinman, R. M., & GRANELLI PIPERNO, A. (1992). IL 6 enhances the generation of cytolytic T lymphocytes in the allogeneic mixed leucocyte reaction. Clinical & Experimental Immunology, 89(1), 148-153.

Mohapatra, J. K., Subramaniam, S., Pandey, L. K., Pawar, S. S., De, A., Das, B., Sanyal, A., & Pattnaik, B. (2011). Phylogenetic structure of serotype A foot-and-mouth disease virus: global

diversity and the Indian perspective. Journal of General Virology, 92(4), 873-879.

O'Neill, L. A., Golenbock, D., & Bowie, A. G. (2013). The history of Toll-like receptors [mdash] redefining innate immunity. Nature Reviews Immunology, 13(6), 453-460.

Ouyang, X., Negishi, H., Takeda, R., Fujita, Y., Taniguchi, T., & Honda, K. (2007). Cooperation between MyD88 and TRIF pathways in TLR synergy via IRF5 activation. Biochemical and biophysical research communications, 354(4), 1045-1051.

Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., & Gray, T. (1995). How to measure and predict the molar absorption coefficient of a protein. Protein science, 4(11), 2411-2423.

Pasquale, A. D., Preiss, S., Silva, F. T. D., & Garçon, N. (2015). Vaccine Adjuvants: from 1920 to 2015 and Beyond. Vaccines, 3(2), 320-343.

Petrovsky, N., & Aguilar, J. C. (2004). Vaccine adjuvants: current state and future trends. Immunology and cell biology, 82(5), 488-496.

Pfaff, E. M. B. O., Mussgay, M., Böhm, H. O., Schulz, G. E., & Schaller, H. (1982). Antibodies against a preselected peptide recognize and neutralize foot and mouth disease virus. The EMBO journal, 1(7), 869.

Ramirez-Carvajal, L., & Rodriguez, L. L. (2015). Virus-resistant pigs might help to stem next outbreak. Elife, 4, e09790.

Ramon, G. (1924). Anatoxine diphthérique. Ann. de l'Inst. Pasteur, 36, 1925.

Reed, S. G., Orr, M. T., & Fox, C. B. (2013). Key roles of adjuvants in modern vaccines. Nature medicine, 19(12), 1597-1608.

Rodriguez, L. L., & Grubman, M. J. (2009). Foot and mouth disease virus vaccines. Vaccine, 27, D90-D94.

Sáiz, M., Núñez, J. I., Jimenez-Clavero, M. A., Baranowski, E., & Sobrino, F. (2002). Foot-and-mouth disease virus: biology and prospects for disease control. Microbes and infection, 4(11), 1183-1192.

Singh, M., & T O'Hagan, D. (2003). Recent advances in veterinary vaccine adjuvants. International journal for parasitology, 33(5), 469-478.

Sivori, S., Falco, M., Della Chiesa, M., Carlomagno, S., Vitale, M., Moretta, L., & Moretta, A. (2004). CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. Proceedings of the National Academy of Sciences of the United States of America, 101(27), 10116-10121.

Soares, H., Waechter, H., Glaichenhaus, N., Mougneau, E., Yagita, H., Mizenina, O., Dubziak, D., Nussenzweig, M. C., & Steinman, R. M. (2007). A subset of dendritic cells induces CD4+ T cells to produce IFN-ɣ by an IL-12 - independent but CD70-dependent mechanism *in vivo*. The Journal of experimental medicine, 204(5), 1095-1106.

Tam, J. P., & Lu, Y. A. (1989). Vaccine engineering: enhancement of immunogenicity of synthetic peptide vaccines related to hepatitis in chemically defined models consisting of T-and B-cell epitopes. Proceedings of the National Academy of Sciences, 86(23), 9084-9088.

Tan, R. S., Ho, B., Leung, B. P., & Ding, J. L. (2014). TLR cross-talk confers specificity to innate immunity. International reviews of immunology, 33(6), 443-453.

Trinchieri, G., & Sher, A. (2007). Cooperation of Toll-like receptor signals in innate immune defence. Nature Reviews Immunology, 7(3), 179-190.

Tripathi, N. K., Shrivastva, A., Biswal, K. C., & Rao, P. L. (2009). METHODS: Optimization of culture medium for production of recombinant dengue protein in *Escherichia coli*. Industrial Biotechnology, 5(3), 179-183.

Tross, D., Petrenko, L., Klaschik, S., Zhu, Q., & Klinman, D. M.

(2009). Global changes in gene expression and synergistic interactions induced by TLR9 and TLR3. Molecular immunology, 46(13), 2557-2564.

Ulanova, M., Tarkowski, A., Hahn-Zoric, M., & Hanson, L. Å. (2001). The common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. Infection and immunity, 69(2), 1151-1159.

van der Burg, S. H., Bijker, M. S., Welters, M. J., Offringa, R., & Melief, C. J. (2006). Improved peptide vaccine strategies, creating synthetic artificial infections to maximize immune efficacy. Advanced drug delivery reviews, 58(8), 916-930.

Whitmore, M. M., DeVeer, M. J., Edling, A., Oates, R. K., Simons, B., Lindner, D., & Williams, B. R. (2004). Synergistic activation of innate immunity by double-stranded RNA and CpG DNA promotes enhanced antitumor activity. Cancer research, 64(16), 5850-5860.

Webb, M. (1968). The influence of certain trace metals on bacterial growth and magnesium utilization. Microbiology, 51(3), 325-335.

Wong, H. T., Cheng, S. C. S., Chan, E. W. C., Sheng, Z. T., Yan, W. Y., Zheng, Z. X., & Xie, Y. (2000). Plasmids encoding foot-and-mouth disease virus VP1 epitopes elicited immune

responses in mice and swine and protected swine against viral infection. Virology, 278(1), 27-35.

Zhang, L., Zhang, J., Chen, H. T., Zhou, J. H., Ding, Y. Z., & Liu, Y. S. (2011). Research in advance for FMD novel vaccines. Virology journal, 8(1), 1.

Zhu, Z. B., Xi, Y., Hu, B., Li, C., Lu, H., Jin, K., Ren, J., Liu, C., Jin, M., & Jin, N. (2012). Immunogenicity of Foot and Mouth Disease Virus Type Asia 1 Protein VP1-2A Fused with a Multi-Epitope Expressed in *Pichia pastoris*. Journal of Animal and Veterinary Advances.

VII. Summary in Korean

구제역은 우제류에서 발생하는 대한민국 제1종 가축전염병이다. 2011년 발생한 구제역이 한국 농가에 큰 피해를 끼치며 국내에서도 구제역 방어 백신 개발이 이루어지고 있으며 특히 기존 약독화/불활화백신이 갖는 장기간 배양 공정, 바이러스 누출로 인한 위험성의 단점을 극복하기 위한 아단위 백신 연구가 진행되고 있다. 아단위 백신, 그 중에서도 멀티-에피톱 백신은 생산 과정의 안전성과 경제성을 개선하였음에도 불구하고 바이러스 일부만을 이용하기 때문에 기존 백신보다 낮은 면역원성을 극복하기 위해 면역증강제의 이용이 필수적이라고 할 수 있다.

본 연구는 고효율 미생물 발현시스템을 이용한 효과적인 가축백신 개발을 위한 세부 전략으로 미생물 성장 변형 배지 개발, 면역증강제 후보로 바이러스 유사 분자 패턴을 갖는 TLR 아고니스트인 CpG ODN과 poly I:C 의 시너지 효과 검정 그리고 백신과 면역증강제 입자화를 통해 최종적으로 구제역 바이러스를 방어하기 위한 백신의 면역 반응 증강을 도모하고자 하였다.

먼저 실험실 수준에서 이용되는 상용 배지의 단점을 극복하기 위한 변형 배지를 고안하였으며, 변형배지인 T2B 에서 향상된 세포 및 단백질 수율을 기록하였다. 그다음으로는 면역증강제로서 TLR 아고니스트의 시너지효과를 검정하고자 하였으며, *in vitro*에서 세포 증식과 전염증 및 항염증 싸이토카인을 분비하는데 있어서 CpG ODN과 와 poly I:C의 시너지 효과를 확인하였다. 이온성 상호작용을 이용하여 입자를 형성한 후 쥐에서 백신접종을 수행한 결과, CpG ODN과 poly I:C를 함께 면역증강제로 이용한 경우 향상된 T 세포 및 B 세포 면역 반응을 이끌어 낼 수 있었다.